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14. ABSTRACT

We have made good progress during the current funding period. We established protein expression systems to generate human and mouse B7-H3 fusion proteins and human and mouse B7x fusion proteins. We then used these fusion proteins to immunize B7-H3 gene knock mice and B7x gene knock-out mice to generate monoclonal antibodies. We successfully generated 13 monoclonal antibodies to B7x and 6 monoclonal antibodies to B7-H3. After functional screen, we will find blocking monoclonal antibodies against B7x or B7-H3 for in vivo immunotherapy for prostate cancer. To further translate our mouse studies to clinical setting, we have started to generate humanized NSG mice for further immunotherapy with human prostate cancer lines and human immune system. In addition, we are testing how B7x and B7-H3 regulate T cell functions and MDSC development. Finally, we have recently discovered the newest immune checkpoint HHLA2 and shown HHLA2 was over-expressed in 3 out 9 human prostate cancer patients.

15. SUBJECT TERMS

B7x; B7-H3; Immune checkpoints; prostate cancer; monoclonal antibodies; immunotherapy.

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1. INTRODUCTION

The B7-CD28 family of ligands and receptors play important roles in T cell costimulation and coinhibition. Phylogenetically they can be divided into three groups. Antibodies blocking these interactions in the group I and II of B7–1/B7–2/CTLA-4 and PD-L1/PD-L2/ PD-1 have had remarkable clinical success in several cancers and are less toxic than traditional chemotherapy. Even though only a small proportion of patients respond to checkpoint blockade, the duration of such responders due to immunological memory is remarkable and is longer than would be expected with any other agent in refractory disease The recent discovery of the new molecules [B7-H3 (CD276), B7x (B7-H4/B7S1) and HHLA2 (B7H7/B7-H5)/TMIGD2 (IGPR-1/CD28H)] of the group III has expanded therapeutic possibilities for the treatment of human diseases. We have discovered the newer members of T cell costimulatory/coinhibitory B7 family, such as B7x and B7-H3, and found that both B7x and B7-H3 down-regulates immune responses via negative T cell costimulation (coinhibition). Our central hypothesis of this grant is that blockade of B7x and B7-H3 generates therapeutic tumor immunity against prostate cancer. We finished the largest investigation of B7 family molecules in human malignancy with 823 prostatectomy patients. This study reveals that prostate cancer patients with strong expression of B7x or B7-H3 by tumor cells are significantly more likely to have disease spread at time of surgery, and are at significantly increased risk of clinical cancer recurrence and cancer-specific death. These basic studies and clinical observations have formed the foundation that targeting B7x and/or B7-H3 could be developed as novel immunotherapies against human prostate cancer. The first aim of this proposal is, therefore, to develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3. The second aim of this proposal is, therefore, to examine combination therapy of anti-B7x/anti-B7-H3 with other therapies. The understanding of these two novel pathway is anticipated to provide new targets for therapeutic interventions that will aid the growing numbers of prostate cancer patients. In addition, it is expected that the proposed research will fundamentally advance the fields of T cell coinhibition in cancer.

2. KEYWORD

B7x; B7-H3; Immune checkpoints; prostate cancer; mAbs; immunotherapy.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3 (Months 1-36).

1a.Generation of mAbs to human and mouse B7-H3 (Months 1-12).

1b.Effectiveness of B7x and/or B7-H3 blockade in a subcutaneous prostate tumor model (Months 6-24).

1c.Effectiveness of B7x and/or B7-H3 blockade in treatment of prostate tumor metastasis (Months 12-30).

1d.Effectiveness of B7x and/or B7-H3 blockade in treatment of primary prostate tumor (Months 12-36).

1e.Effect of B7x- or B7-H3-specific mAbs on T cell function in vivo in humanized NSG mice (Months 12-24).

Task 2. Examine combination therapy of anti-B7x/anti-B7-H3 with other therapies (Months 12-36).

2a.Synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4 (Months 18-30).

2b.Synergy between B7x/B7-H3 blockade and regulatory T cell depleting (Months 18-30).

2c.Potential mechanism of anti-B7x/B7-H3 therapy: blockade of B7-mediated T cell immunosuppression (Months 12-36).

2d.Potential mechanism of anti-B7x/B7-H3 therapy: effect on the generation of induced regulatory T cells (iTreg) and myeloid-derived suppressor cells (MDSCs) (Months 12-36).

2e.Potential mechanism of anti-B7x/B7-H3 therapy: antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (Months 12-30).

What was accomplished under these goals?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3 (Months 1-36).

- We generated B7x gene knock-out mice. Therefore we took advantage of these mice for generating mAbs against both human and mouse B7x. Briefly, B7x knock-out mice were immunized with 100ug of human B7x-Ig fusion protein, after 3 weeks the mice were immunized with 100 ug of mouse B7x-Ig fusion protein with hopes to produce cross-reactive antibodies. Two immunized mice generated good anti-B7x antibodies in sera, suggesting our immunization protocol worked well. These two mice were finally boosted with 100 ug of human B7x- Ig. Four days after the final boost spleens were harvested and fused to a myeloma cell line and plated over 66 plates in preparation for a primary screening.
- ELISA is the traditional method that is used to screen positive wells for individual clones, but our lab has recently developed a FACS based protocol to perform the screening that is much faster and more suitable for our purposes. For example we

would use 3T3 cells expressing hB7x tagged to YFP and 3T3 cells expressing another protein that is YFP negative. We added these cells in a 50/50 ratio to an individual well than added supernatant from one of the many potential clones to the well for 30 minutes which we hoped to contain antibodies against B7-H3. Then we added a conjugated anti-mouse Ig for 30 minutes and went to FACS to look for positive hits.

- We went through and screened all plates and sub-cloned each positive hybridoma 3 times to ensure a monoclonal population. We were able to obtain a total of 12 different clones (Fig 1.). We have purified all these 12 anti-B7x mAbs and are performing immunotherapy against prostate cancer lines in mice in vivo.

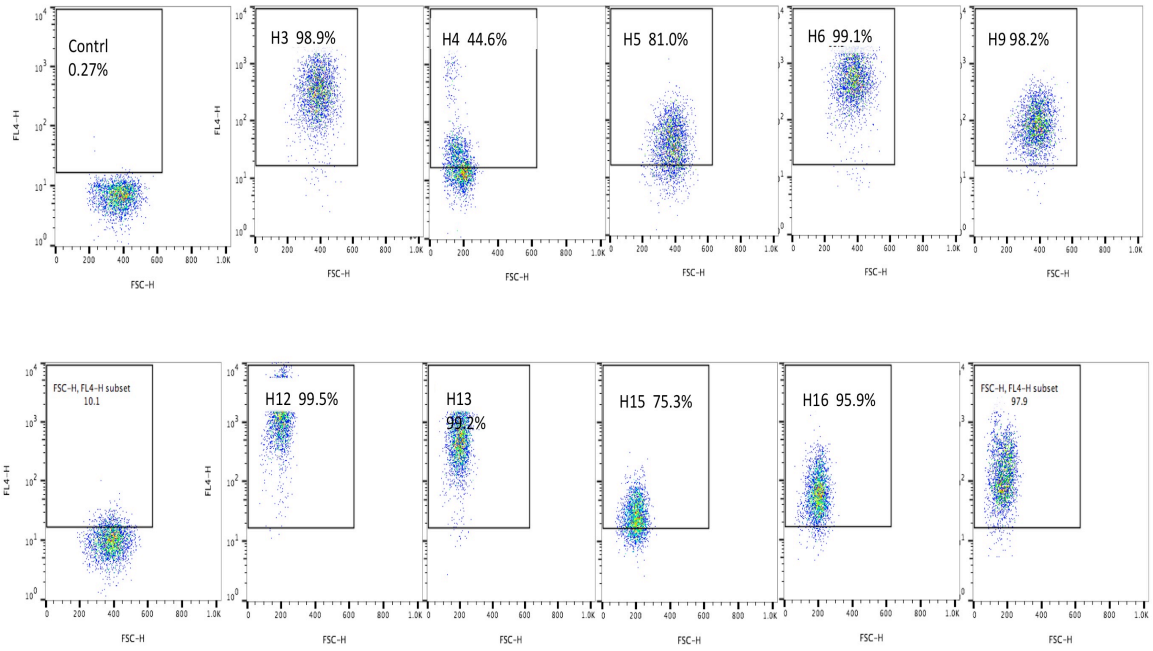

Figure 1. Total 12 anti-B7x mAbs were generated. B7x-/- mice were generated. B7x-Ig fusion were produced and purified from the S2 expression system. B7x-/- mice were immunized with B7x-Ig fusion proteins and then spleens were harvested and fused to establish mAbs.

- Similarly, we generated B7-H3 gene knock-out mice. Therefore we took advantage of these mice for generating mAbs against both human and mouse B7-H3. Briefly, B7-H3 knock-out mice were immunized with human B7-H3-Ig fusion protein and spleens were harvested and fused to establish mAbs. We are still in the process of screening and subcloning. At present, we have 6 IgG type mAbs: 3 IgG1, 1 IgG2a, and 2 IgG2b (Fig. 2). In addition, we have at least 6 mAbs which are still the mix of IgG/IgM and need to further subcloning (Fig. 2).

We will purfy these mAbs and use them in vivo immunotherapy against prostate cancer lines in mice.

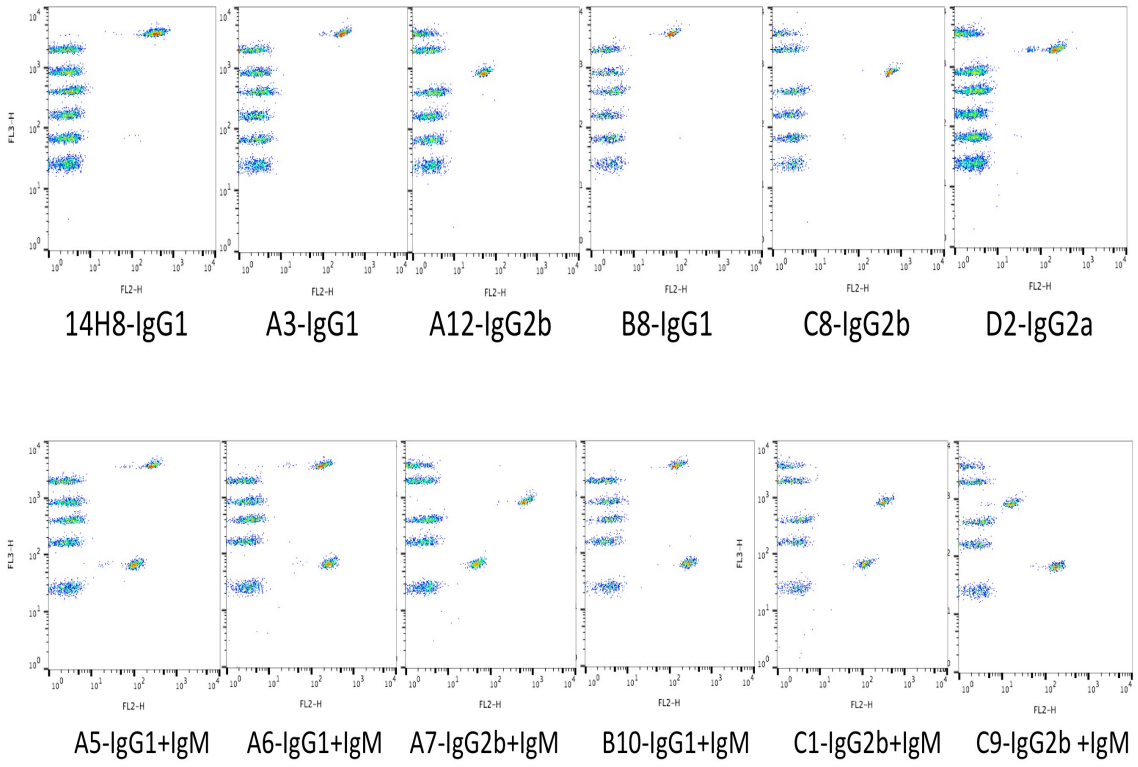

Figure 2. Anti-B7-H3 mAbs were generated. B7-H3-/- mice were generated. B7-H3-Ig fusion were produced and purified from the S2 expression system. B7-H3-/- mice were immunized with B7-H3-Ig fusion proteins and then spleens were harvested and fused to establish mAbs. we have 6 IgG type mAbs and at least 6 mAbs which are still the mix of IgG/IgM and need to further subcloning.

- To facilitate future clinical trails, we want to examine the effect of B7x- or B7-H3-specific mAbs on T cell function in vivo in humanized NOD-scid IL2Rg-/- mice (NSG). We have successfully established humanized NSG mice for prostate cancer immunotherapy. NSG mice were first engrafted with human prostate cancer lines and then human peripheral blood mononuclear cells. These humanized mice usually develop T cell-mediated graft-versus host immune responses within one month, so human T cells are activated and express receptors for B7x and B7-H3 and human prostate cancer cells express B7x or B7-H3. FACS showed the approach how we identified human prostate cancer cells from these NOG mice (Fig. 3). These mice will be used to examine if anti-B7x and/or anti-B7-H3 enhance T cell function and reduce prostate cancer growth in vivo.

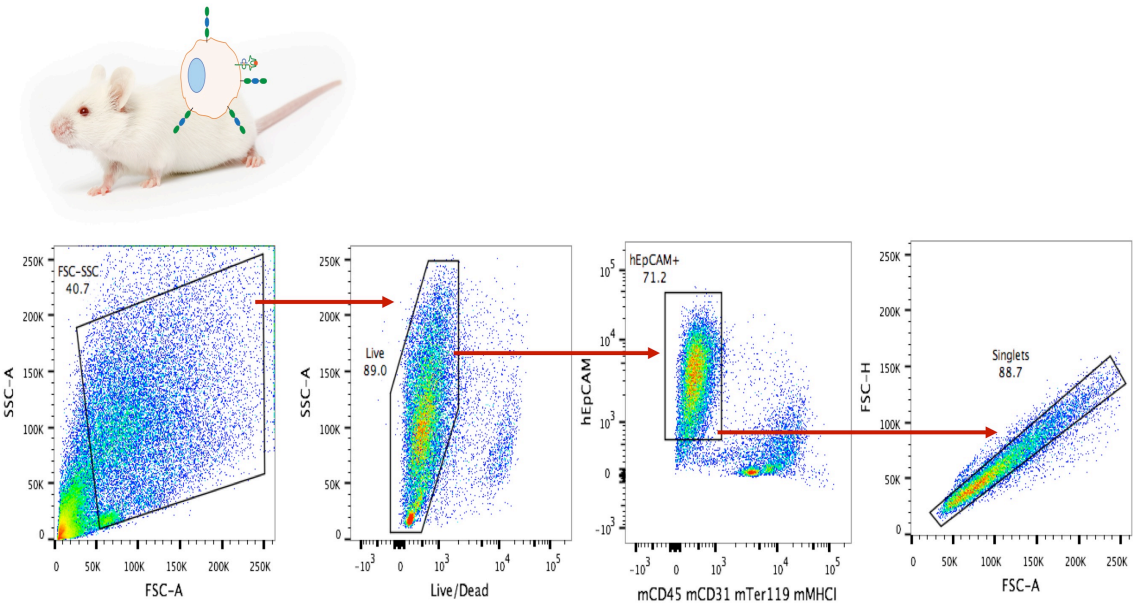

Figure 3. Establishment of humanized NSG mice for prostate cancer immunotherapy. FACS showed the approach to identify human prostate cancer cells from these NOG mice. Human cancer cells were hEpCAM+mCD45-mCD31-mTer119-mMHCI-.

Task 2. Examine combination therapy of anti-B7x/anti-B7-H3 with other therapies (Months 12-36).

- We have screened a panel of mAbs against PD-1 or CTLA-4 for in vivo immunotherapy. Our results showed that clone RMPI–14 of anti-PD-1 and clone 9H10 of anti-CTLA-4 worked well in vivo. Therefore we are performing experiments to see if there are synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4.
- We are performing experiments to dissect the potential mechanisms of anti-B7x/B7-H3 therapy. We wanted to see if B7x affects the differentiation of naïve CD4 T cells into Th1, Th2, Th9, Th17, and iTregs. Naïve CD4 T cells were incubated in the presence of B7x-Ig with the following conditions:
  Th0 (CD3/CD28)
  Th1 (CD3/CD28 + anti IL-4 + IL-2)
  Th2 (CD3/CD28 + anti IFNg + IL-4)
  Th9 (CD3/CD28 + TGF beta + IL-4 + IL-2 + anti IFNg)
  Th17 (CD3/CD28 + IFNg + IL-4 + IL-6 + TGF beta)
  iTregs (CD3/CD28 + TGF beta + IL-2)
  On day 5, CD4 T cells were determined for the expression of IFN-g (Th1), IL-4 (Th2), IL-17 (Th17), and Foxp3 (Treg). Our preliminary results suggest that B7x is able to inhibit Th1 T cells (Fig. 4).

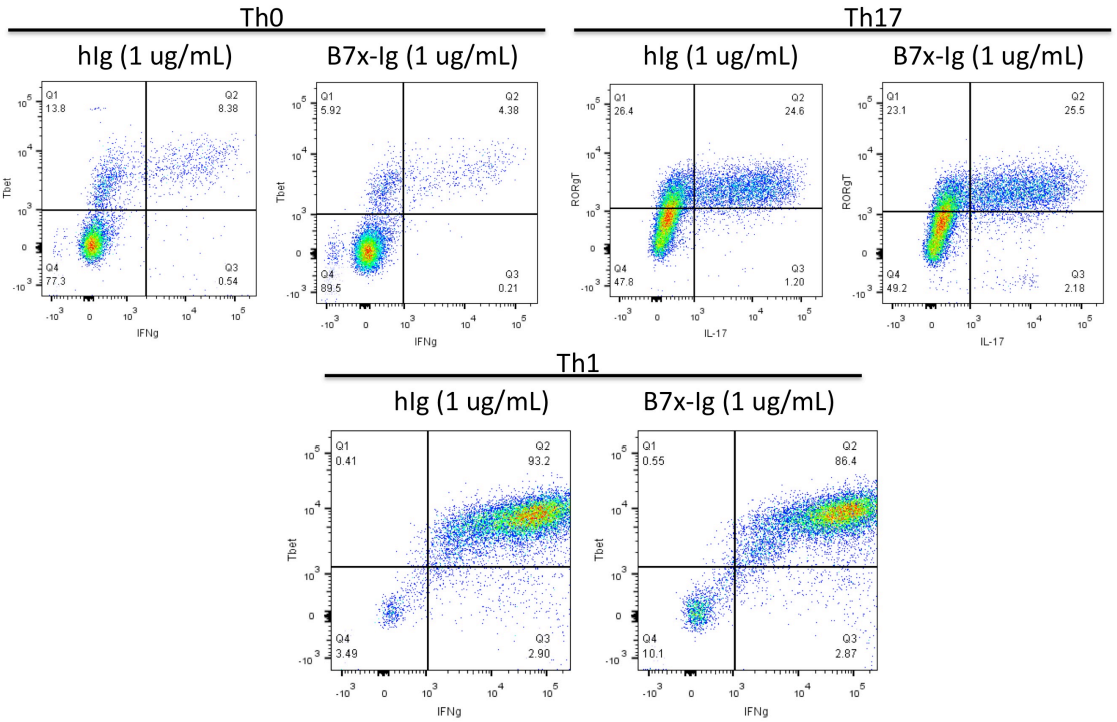

Figure 4. Effects of B7x on the differentiation of naïve CD4 T cells into Th1 and Th17. Naïve CD4 T cells were incubated in the presence of B7x-Ig with the following conditions: Th0 (CD3/CD28), Th1 (CD3/CD28 + anti IL-4 + IL-2), and Th17 (CD3/CD28 + IFNg + IL-4 + IL-6 + TGF beta). Day day 5, CD4 T cells were determined.

- WE recently discovered HHLA2 as the newest B7 immune checkpoint and it was over-expressed in human prostate cancer. We further generated new mAbs to HHLA2 and developed a new IHC protocol for HHLA2 protein expression. Interestingly enough, none of three normal prostate tissues expressed HHLA2, whereas three out of nine prostate cancer samples were HHLA2 positive (Fig. 5), suggesting the HHLA2 pathway represents a novel immunosuppressive mechanism within the tumor microenvironment of human prostate cancer and an attractive target for human prostate cancer therapy.

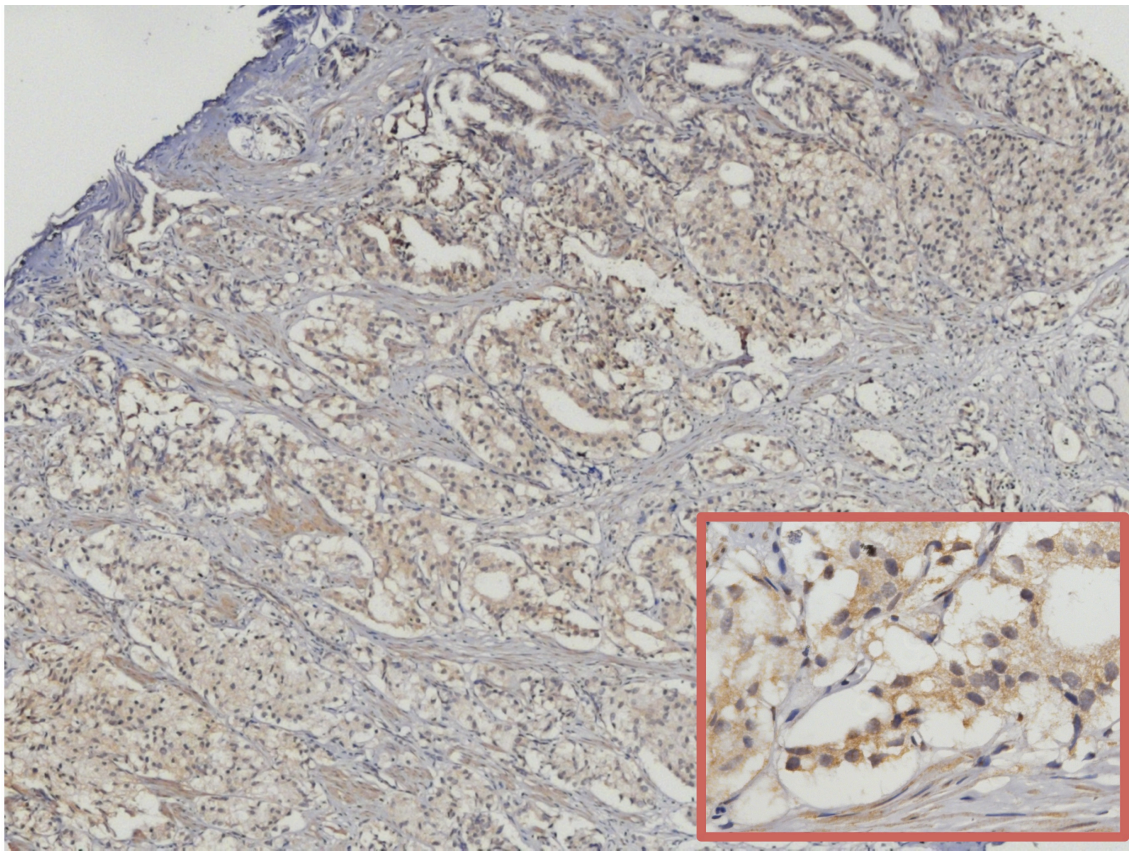

Figure 5. HHLA2 is the newest B7 immune checkpoint and is over-expressed in human prostate cancer.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We will 1) further dissect the mechanisms of antiB7x/B7-H3 therapies; 2) test there are synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4; and 3) identify receptors for B7x.

4. IMPACT

Nothing to Report

5. CHANGES/PROBLEMS

None

6. PRODUCTS (Publications)

1) Assal A, Kaner J, Pendurti G, **Zang X**. Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1. ***Immunotherapy***, 7:1169-1186, 2015.
2) Picarda E, Ohaegbulam KC, **Zang X**. Molecular pathways: Targeting B7-H3 (CD276) for human cancer immunotherapy. ***Clinical Cancer Research***, 22:3425-3431, 2016
3) Yao Y, Ye H, Qi Z, Mo L, Yue Q, Baral A, Hoon DSB, Vera JC, Heiss JD, Chen CC, Hua W, Zhang J, Jin K, Wang Y, **Zang X***, Mao Y*, Zhou L*. B7-H4(B7x)-mediated cross-talk between glioma-initiating cells and macrophages via the IL-6/JAK/STAT3 pathway lead to poor prognosis in glioma patients. ***Clinical Cancer Research***, 22:2778-2790, 2016 (*Co-corresponding authors)
4) Liu W, Almo SC, **Zang X**. Co-stimulate or co-inhibit regulatory T cells, which side to go? ***Immunological Investigations***, 45:813-831, 2016
5) Janakiram M, Pareek V, Cheng H, Narasimhulu D, **Zang X**. Immune checkpoint blockade in human cancer therapy: Lung cancer and hematopoietic malignancy. ***Immunotherapy***, 8:809-819, 2016
6) Janakiram M, Shah UA, Liu W, Zhao A, Schoenberg MP, **Zang X**. The third group of B7-CD28 immune checkpoint family: HHLA2, TMIGD2, B7x and B7-H3. ***Immunological Reviews***, accepted.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

PI: Xingxing Zang

Kim Ohaegbulam, Elodie Picarda, Qizhe Sun, Xiaoshen Dong

No Change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

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Review

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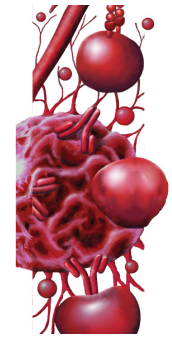
Immunotherapy



Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1

Manipulation of co-stimulatory or co-inhibitory checkpoint proteins allows for the reversal of tumor-induced T-cell anergy observed in cancer. The field has gained credence given success with CTLA-4 and PD-1 inhibitors. These molecules include immunoglobulin family members and the B7 subfamily as well as the TNF receptor family members. PD-L1 inhibitors and LAG-3 inhibitors have progressed through clinical trials. Other B7 family members have shown promise in preclinical models. TNFR superfamily members have shown variable success in preclinical and clinical studies. As clinical investigation in tumor immunology gains momentum, the next stage becomes learning how to combine checkpoint inhibitors and agonists with each other as well as with traditional chemotherapeutic agents.

Keywords: B7 family • checkpoint proteins • immunotherapy • TNFR superfamily • translational medicine

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One of the hallmarks of cancer is the ability of the malignant cell to escape eradication by the immune system [1]. Proposed over a century ago, the concept of immune control of cancer continues to develop [2,3]. The existence of tumor antigens led Burnett and Thomas to form their hypothesis about cancer immune surveillance where the adaptive immune system was responsible for preventing the development of cancer in immunocompetent hosts. This hypothesis fell out of favor until the 1990s when improved mouse models of immunodeficiency were developed and particularly when the role of IFN-γ in promoting immune-mediated rejection of transplanted tumor in mice [4].

Tumors are variably infiltrated by cytotoxic T lymphocytes (CTLs), but a dense infiltration portends a better prognosis [5–7]. The T-cell response follows a complex interaction between an antigen-presenting cell (APC) and a T cell. TCR recognition of an antigen on MHC molecule is not sufficient, a second signal provided by a member of the B7 family is required [8]. CD28 provides the primary co-stimulatory signal for the activation of T cells after it engages B7-1 (CD80) or B7-2 (CD86) [9]. CTLA-4 is a CD28 homologue that interacts with B7-1 and B7-2 and, in contrast to CD28, provides an inhibitory signal [10,11]. Newly identified members of the B7 family also provide inhibitory signals the roles of which continue to be explored [12]. Blocking CTLA-4 mediated inhibition of the T-cell effector response has been an attractive therapeutic target. Monoclonal antibodies (mAb) that block CTLA-4 are effective in mouse models of a variety of tumors [13–15]. Ipilumumab (Yervoy®) is US FDA approved for the treatment of metastatic malignant melanoma and represents the first success story of T-cell checkpoint inhibitor immunotherapy [16].

A more recent success story in cancer immunology is that of PD-1. PD-1 was first identified in lymphoid cells lines induced to undergo programmed cell death [17]. Later reports noted that PD-1 is expressed on activated T and B cells, dendritic cells (DCs) and monocytes upon stimulation where it is found to play an inhibitory role [18–20]. PD-1 is highly expressed on T cells and leads to

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T cell exhaustion [21,22]. PD-1 expression is also noted on $CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) where it contributes to their inhibitory role [23]. Several mAbs targeting PD-1 have progressed through clinical trials. The first FDA approved mAb was pembrolizumab (Keytruda®), also known as lambrolizumab after showing response rates in melanoma patients who have progressed after first line therapy including immunotherapy with ipilimumab (KEYNOTE-001 trial) or in comparison to investigator-choice chemotherapy (KEYNOTE-002) [24,25]. The second PD-1 targeting mAb to receive FDA approval was nivolumab (Opdivo®). It was well tolerated in Phase I studies in solid tumors as well as lymphomas [26–30]. Similar to pembrolizumab, nivolumab was shown to be superior to chemotherapy in the second line setting in melanoma in the Phase III CheckMate-037 trial [31,32]. Nivolumab yielded better survival and higher response rated in comparison to docetaxel in the treatment of advanced squamous non-small-cell lung cancer [33]. Other PD-1 mAbs include pidilizumab (CT-011) which was the first one to reach clinical trials and remains in development (studies reviewed in [34]) and more recently MEDI0680 which is entering clinical trials [35,36].

Blocking CTLA-4 and PD-1 are not the exclusive path toward T-cell 'dis-inhibition'. A variety of immunomodulatory pathways have been studied and exploited clinically with varying degrees of success and are at different stages of clinical development. Other members of the B7 family, part of the immunoglobulin superfamily, include B7x, HHLA2 and B7-H3 which play an inhibitory role. VISTA, Tim-3 and LAG-3 are members of the immunoglobulin superfamily have also been shown to play an inhibitory role. Immunomodulatory pathways include members of the TNF receptor family and their ligands which have been studied as targets for cancer immunotherapy. These inhibitory and stimulatory molecules that have been studied as therapeutic targets are depicted in Figure 1A & B, respectively. Finally, indoleamine 2,3–dioxygenase 1 inhibitors have also been studied as antitumor therapies as discussed below.

PD-L1 & PD-L2

The first reported ligand for PD-1 is PD-L1 (B7-H1) with wide expression at the mRNA level in lymphoid and nonlymphoid tissues [37]. It is a cell surface protein that is expressed on activated APC, T and B lymphocytes and other cells. It inhibits TCR mediated T-cell proliferation and cytokine production through the engagement of PD-1 [38]. The PD-1/PD-L1 interaction induce T-cell tolerance in lymphoid tissue before their exit to the periphery, and blockade of this interaction can reverse T-cell anergy [39]. Additionally, PD-L1 expressed on tumor cells can also act as a ligand to deliver an anti-apoptotic signal that leads to resistance to cytolytic function of CTL as well as to Fas-induced and drug-induced apoptosis [40]. Another interesting fact is that B7-1 was also shown to interact with PD-L1 which results in inhibition of T cells [41,42]. A second ligand for PD-1 is PD-L2 (B7-DC), which inhibits TCR mediated T-cell proliferation and cytokine production [43,44]. It is mainly expressed on DCs and macrophages [44,45]. Recently, a novel binding partner for PD-L2 is identified named RGMb, which is important for the development of respiratory immune tolerance [46].

PD-L1 is expressed in a variety of human carcinoma specimens as well as hematological malignancies such as multiple myeloma, leukemia and peripheral T-cell lymphoma and has been correlated to poor prognosis [8,34]. Several mAbs that target PD-L1 have reached clinical trials. BMS–936559 is a fully human monoclonal IgG4 antibody that blocks PD-L1 [47]. Anti-PD-L1 antibodies inhibited tumor growth in murine syngeneic tumor models with a durable antitumor immunity. BMS–936559 can reverse *in vitro* Treg mediated suppression and does not cause antibody-dependent cytotoxicity or complement-dependent cytotoxicity [47]. The first clinical trial with BMS–936559 also demonstrated high tolerability and durable responses [47]. Other monoclonal anti-PD-L1 antibodies include MEDI4736 [48,49], atezolizumab (MPDL3280A) which demonstrated a 43% response rate in a Phase I clinical trial in metastatic urothelial bladder cancer patients resulting in an FDA breakthrough designation [50], and MSB0010718C which exhibits antitumor activity by blocking PD-L1 as well as antibody-dependent cell-mediated cytotoxicity [51,52].

B7x (B7-H4/B7- S1)

B7x is an inhibitory transmembrane protein that binds activated T cells and is a member of the B7 family [53–55]. It inhibits CD4 and CD8 cell proliferation and cytokine production [53]. It is hardly expressed on professional APC but is expressed on nonlymphoid tissues, mainly epithelial tissues where a role in immune tolerance is postulated [54,56–58]. It is expressed in the lung epithelium and is implicated in attenuating the immune response to bacterial infection in mice [56]. B7x is expressed in a variety of human cancers which include cancers of the brain, esophagus, lung, breast, pancreas, kidney, gut, skin, ovary and prostate [59]. Prostate cancer specimens from patients treated with radical prostatectomy had 15% prevalence of B7x expression and high expression was significantly associated with a higher risk of prostate cancer related death [60]. B7x expression in renal cell carcinoma is

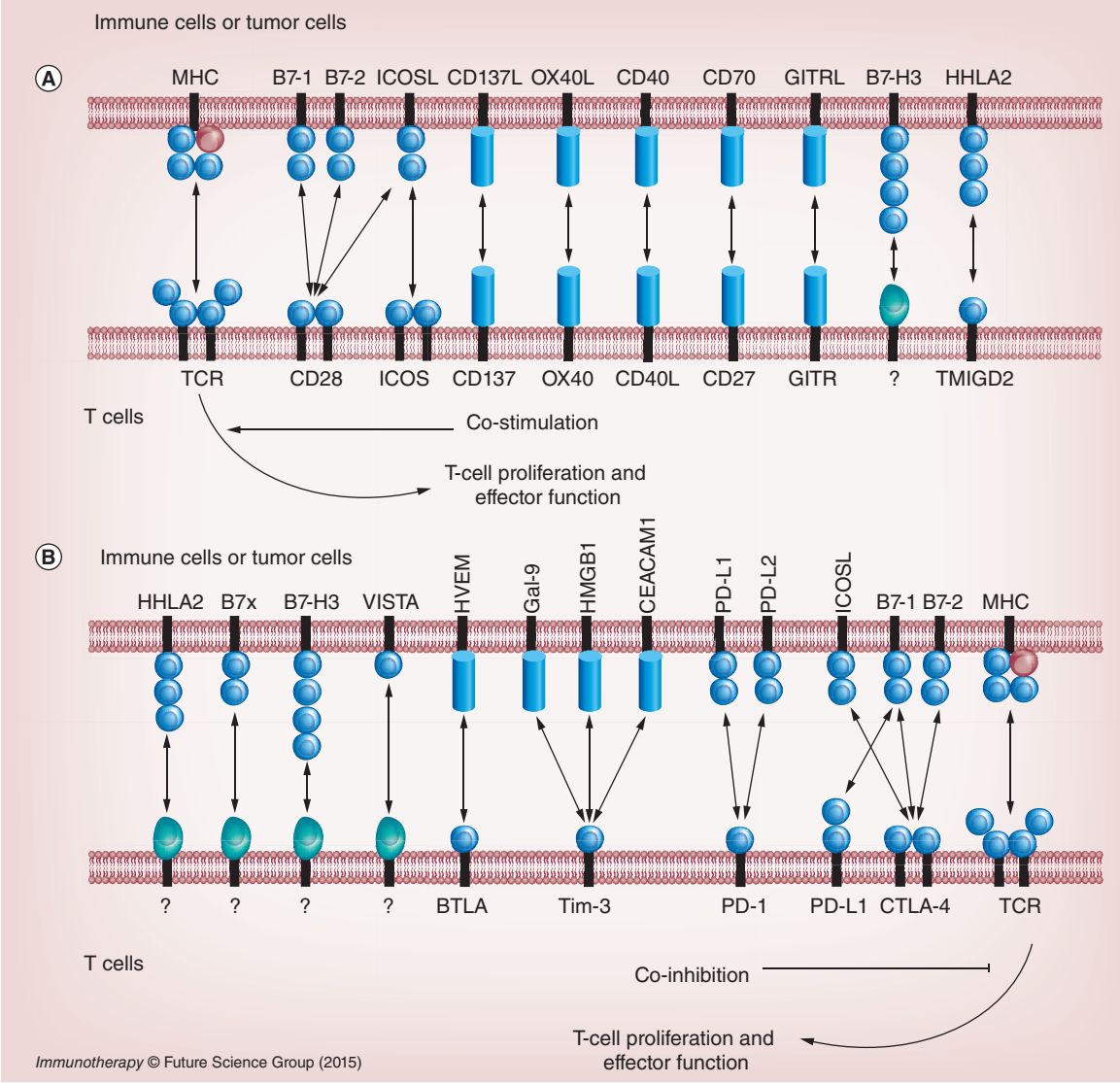

Figure 1. Summary representation of T-cell molecules. (A) Summary representation of T-cell co-stimulatory molecules. **(B)** Summary representation of T-cell co-inhibitory molecules.

associated with adverse clinical and pathological features as well as poor survival [61]. Tumor expression of B7x in human gastric cancer predicts poor survival [62]. Similar findings were also reported in studies of ovarian cancer and lung cancer [63,64]. In a preclinical model, mouse colon carcinoma cells line CT26 transfected with murine or human B7x resulted in a higher number of lung metastasis and shorter survival [65]. Blockade of B7x with a mAb resulted in a reduction of number of lung metastasis in a CT26 as well as 4T1 based mouse models of lung metastasis [65]. B7x thus represents a very promising target for cancer immunotherapy.

HHLA2 (B7y/B7-H5/B7H7)

HHLA2 is another member of the B7 family that modulates T-cell function [66,67]. It is expressed on monocytes and induced on CD19 positive B cells. HHLA2–Ig fusion protein bound resting and activated CD4 and CD8 T cells, as well as APC. It was shown to inhibit proliferation of CD4 and CD8 T cells in the presence of TCR signaling as well as T-cell cytokine production [66]. TMIGD2, also called CD28H or IGPR–1, is identified as one of the receptors for HHLA2 [67,68]. IGPR–1 was initially reported to be an adhesion molecule involved in angiogenesis [68]. HHLA2 expression in non-lymphoid tissues was limited to placenta, GI tract, kidney, gallbladder and breast, but its expression was more common in human tumor specimens including breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney and esophagus [68]. In a cohort of 50 patients with triple negative breast cancer, 56% of patients had HHLA2 expression

on their tumors, and high HHLA2 expression was significantly associated with regional lymph node metastasis and stage. Of interest, increase in HHLA2 expression was also due to an increase in gene copy number, and not just stimulation [68]. There is much to be discovered about HHLA2 and it represents a potential target for cancer immunotherapy.

B7-H3

B7-H3 was first identified as a molecule that binds a receptor on activated T lymphocytes [69]. Its expression was inducible on DCs and was initially thought to be costimulatory to T lymphocytes [69]. *In vivo* studies in mouse models showed that B7-H3 was an inhibitory to T lymphocytes and preferentially inhibits T helper cells type 1 response [70]. The receptor for this ligand is still unclear. It is expressed in some cancer cells and was associated with regional nodal metastasis [12,71]. Currently the majority of evidence suggests that this is a co-inhibitory ligand for T-cell response [72]. B7-H3 was found to be upregulated in graft-versus-host disease (GVHD) target organs and its absence in B7-H3$^{-/-}$ mice resulted in augmented GVHD lethality and T-cell proliferation and function [73]. Increased B7-H3 expression in cancer specimens has been reported [74], and has been correlated to worse outcomes [60,75]. Therefore, B7-H3 is another potential target for cancer immunotherapy.

VISTA

VISTA is a recently discovered negative modulator of the immune system [76]. VISTA is primarily expressed on hematopoietic cells, including APCs and T cells [77]. It is a suppressor of CD4 and CD8 T cells. In addition, within the CD4 subset, both effector and memory T-cells are effectively suppressed. T cells cultured with soluble VISTA-Ig fusion protein show no shift in expression of CD45RA to CD45RO [77]. Another notable phenomenon is that, while it blocks proliferation, it does not induce apoptosis and thus the cells remain viable. Lastly, the suppressive effect on T cells appears to be long lasting, even after VISTA effect was removed. In addition to being immunosuppressive of T cells, it is also immuneregulatory. Under neutral conditions, VISTA-Ig is capable of suppressing naïve T-cells from forming Treg. This however was not the case when the culture conditions were changed, and in the presence of IL-2 and anti-CD28 VISTA-Ig was actually able to increase the proportion of Treg. Of note, VISTA does not appear to have any effect *in vitro* on B-cell proliferation or regulation. Its effect on cytokine production included reduced levels of IL-10, IFN-γ and TNF-α [77]. Anti-VISTA mAb exacerbate experimental auto-immune encephalomyelitis as well as enhancing antitumor immune responses [76]. Anti-VISTA mAbs are able to increase the number of tumor specific T cells in the periphery and enhance the infiltration, proliferation and effector function of tumor infiltrating lymphocytes within the tumor microenvironment [76]. In a melanoma model, both transplantable and inducible cancers are suppressed effectively with anti-VISTA monotherapy [78]. VISTA's antitumor effect was also explored in concert with a peptide-based cancer vaccine where VISTA blockade synergistically impaired tumor growth.

CD27

CD27 is a T-cell differentiation antigen and member of the TNFR superfamily [79]. It has increased membrane expression on anti-CD3-activated T cells. Agonistic CD27 mAb resulted in enhanced proliferation of CD3 stimulated T cells. CD27 and its ligand CD70 are thought to have important effects on T-cell function [79]. Using intranasal influenza virus infection as a model system, CD27 has been shown to be a major determinant of CD8 T-cell priming at the site of infection. CD27 signaling, along with other signaling including CD28, is crucial for the generation of antigen specific CD8 T cells. Via cell survival stimulation, CD27 promotes accumulation of activated T cells thereby expanding the proportion of virus specific T cells [79]. The survival signal relies on IL-2R signaling and autocrine IL-2 production and CD27 is responsible for long-term survival of primed CD8 T cells, and hence memory. CD27 function has been extensively studied in mice and is transiently expressed during the germinal center reaction. CD27 expression is most abundant during the phase of expansion of primed B cells and is absent from memory B cells. It appears that CD27 T cells provide help to B cells to form small germinal centers. Additionally, CD27 signaling in B cells results in enhanced levels of plasma cell formation and increased IgG production. Interestingly, constitutive CD27 signaling could have alternate effects on cellular and humoral immunity [79]. Collectively, the data elucidated CD27 signaling as a determinant of germinal center kinetics. In mouse models, costimulatory effect of CD27 was necessary for anti-CD40 antitumor efficacy [80]. Antitumor efficacy was shown in a mouse model of lymphoma using an anti-CD27 mAb. Anti-CD27 demonstrated no effect in SCID mice suggesting the need for an intact adaptive immune response and that the response itself was not due to a direct effect on the lymphoma cells [80].

A fully human anti-CD27 mAb, IF5, increased survival in a mouse model of leukemia and lymphoma [81]. Its toxicity was assessed in a non-human primate model and has entered clinical development under the name

CDX–1127 (Varlilumab) and was assessed in a Phase I clinical trial [82]. The drug was well tolerated and responses included a complete response in a patient with stage IV Hodgkin's lymphoma who had previously failed stem cell transplant, chemotherapy and brentuximab-vedotin and three additional patients with stable disease.

OX40/OX40L

OX40 is a member of the TNFR superfamily and is expressed on activated CD4 and CD8 T cells as well as other lymphoid and nonlymphoid cells [83]. It is also expressed in natural killer (NK) cells, NKT cells and neutrophils. OX40L is also a member of the TNFR superfamily and its expression is inducible on APC. Nonlymphoid cells can also be induced to express OX40L which supports of the role of the OX40/OX40L pathway in regulating the T-cell response. T cells themselves can express OX40L which represents an additional mechanism for T-cell response amplification. TCR signaling is sufficient to induce OX40 in activated CD4 and CD8 T cells, however this is augmented by CD28 and B7-1/B7- 2 interaction and modulated by cytokines such as IL-1, IL-2 and TNF [83]. OX40L expression on the other hand is can be induced on APC upon activation by ligation of CD40 or by Toll-like receptors [83].

The OX40/OX40L pathway plays a large role in T cell expansion and survival, primarily by maintaining later proliferation and T-cell survival through the effector phase [83]. OX40 ligation can also directly inhibit naturally occurring Treg activity in mice providing another means to promoting effector T-cell proliferation and survival [83]. Foxp3 expression on naïve CD4 T cells is blocked by OX40/OX40L activity which supports a role in the suppression of naïve CD4 T-cells differentiation to become Treg. There have been conflicting reports however on the impact of OX40 signaling, which is expressed constitutively expressed on Treg, and may in fact promote Treg responses depending on the cytokine milieu [84].

The rationale for targeting OX40/OX40L signaling for cancer immunotherapy is supported by the expression of OX40 in tumor infiltrating lymphocytes. Preclinical models have shown that injection of agonist OX40L-Ig fusion proteins, OX40 mAb, RNA aptamers that bind OX40 and transfection of tumor cells or DCs with OX40L can all suppress tumor growth [83]. In mouse models of cancer including sarcoma, melanoma and glioma, among others, OX40 activity has been shown to decrease tumor growth [84]. The mechanism of tumor growth suppression is related to CD8 T-cell survival, and/or promotion of CD4 T-cell help for CD8 T cells. There may be an additional role via augmentation of NK cell activity. Tumor infiltrating Tregs express high levels of OX40, and the signaling of OX40 within this environment suppressed their activity [85]. In animal models, stimulation of OX40 appears to both augment antitumor activity as well as suppress Treg activity. Human studies include a Phase I trial using anti-OX40 mab 9B12 in patients with advanced cancers (NCT01644968 [86]) [87]. Therapy was well tolerated with no maximum tolerated dose reached. Most common grade 3 or 4 side effects included lymphopenia that was transient. Best response to therapy by response evaluation criteria in solid tumors (RECIST) only included stable disease with some tumor regression noted but less than 30% of overall tumor. One limitation of this agent was the induction of human antimouse antibodies, which precluded patients from receiving additional cycles. Nevertheless, this study provides evidence in humans that OX40 agonism can augment the immune system by stimulating CD4 and CD8 T-cell proliferation, CD8 IFN-γ production and increased antibody titers and T cell recall in response to tetanus immunization.

It seems that targeting OX40 alone may not be sufficient to elicit a robust antitumor response, and thus combination immunotherapy, particularly with antagonistic anti-CTLA-4 and anti-PD-1 antibodies, has been an area of study in preclinical models [84]. Combination therapies may stimulate complimentary pathways that synergistically respond to poorly immunogenic or large tumors, which has been an area of weakness in immunotherapy. Naturally, synergism may also extend to worsening the toxicity profile of such therapy but the fact the anti-OX40 therapy was fairly well tolerated may be promising.

CD40/CD40L

CD40 and its ligand CD40L are members of the TNFR/TNF family [88]. CD40 is expressed on professional APC as well as other non-immune cells and tumors. Its ligand, CD40L is transiently expressed on T cells and other non-immune cells under inflammatory conditions [88]. Inherited lack of CD40L is responsible for x-linked hyper-IgM syndrome (H-XIM). It activates DCs and allows them to stimulate CD8 T-cell activation and proliferation [89].

Binding of CD40L to CD40 promotes CD40 clustering on the cell surface, as well as recruitment of adapter proteins known as TRAFs to the cytoplasmic domain of CD40 [88]. CD40 signaling is carried forward by different pathways which include MAPKs, NF kappa B, PLC and PI3K. Additionally, it is noted that JAK3 binds the cytoplasmic domain of CD40 and can mediate other cellular processes. CD40/CD40L can directly activate DCs [89]. This CD40/CD40L inter-

action is necessary for the maturation and survival of DCs and CD40-dependent maturation of DCs leads to sustained expansion and differentiation of antigen specific T cells. The increased life span of DCs is very important in driving cell-mediated immunity. Without the CD40 survival signal, passive apoptosis of T cell is induced [88].

CD40 signaling by B cells is required for the generation of high titers of isotype switched high affinity antibody as well as for the development of humoral immune memory [88]. The binding of CD40L on CD4 T cells to CD40 on activated B cells is an important step in initiation and progression of the humoral immune response. Once CD40 signaling is active, there are downstream effects including B-cell intracellular adhesion, sustained proliferation, differentiation and antibody isotype switching. This process is essential for memory B cells and long lived plasma cells. B-cell fate is heavily influenced by CD40 signaling [88]. This signaling via binding of CD40 and CD40L can help determine whether the maturing B cell becomes a plasmablast or seeds a germinal center. With the activated CD40 pathway, the B cell will go on to form a germinal center. The lack of CD40 signaling is sufficient to block germinal center formation. T helper cells are recruited to these germinal centers, some of which express CD40L on their cell surface, which serve to maintain the germinal center. The lack of CD40 signaling in germinal center B cells increase Fas dependent apoptosis.

CD40 is expressed in mouse and human models of melanoma, prostate and lung cancers, and carcinomas of the nasopharynx, bladder cervix and ovary [88]. Hematologic malignancies such as non-Hodgkin's lymphoma, Hodgkin's lymphoma, acute leukemias and multiple myeloma also express CD40. Anti-CD40L mAb treatment inhibits the generation of protective immune responses from potent tumor vaccines [90]. Additionally, using CD40 deficient mice, no protective antitumor immune response was induced following a protective vaccination regime. Early studies using a lymphoma model showed that agonistic anti-CD40 antibodies were able to eradicate tumor. Gene delivery of CD40L to DCs and tumor cells was sufficient to stimulate a long lasting systemic antitumor immune response in a murine model [90]. The approach of gene therapy with adenovirus expressing CD40L has been shown to be successful in colorectal carcinoma, lung carcinoma and melanoma murine models. CD40 agonism alone, however, was not sufficient for antitumor response likely due to the lack of TLR signaling.

Clinically, recombinant CD40L has been used in patients with solid tumors or non-Hodgkin's lymphoma given subcutaneously daily for 5 days in a Phase I clinical trial [91]. Responses to therapy included 6% of patients with a partial response and one patient with a complete response. Humanized agonistic CD40 mAb include CP–870,893, SGN-40 and HCD 122. In a Phase I trial, CP-870,893 produced a partial response in 14% of patients (27% of melanoma patients). Dose-limiting toxicities were observed and included cytokine release syndrome [88]. Dacetuzumab (SGN-40) is a weak agonist and was studied in a Phase I trial in patients with refractory or recurrent B-cell non-Hodgkin's lymphoma [92]. Toxicity was shown to be acceptable and antitumor activity was seen with six objective responses, 13 patients with stable disease. The overall response rate for patients in this cohort was 18%. In a Phase II trial with this drug, 46 patients again with relapsed or refractory diffuse large B-cell lymphoma were treated and the overall response rate was 9% and disease control rate (stable disease or better) was 37% [92]. Lucatumumab (HCD122) is a fully human anti-CD40 antagonist and was tested in 28 patients with refractory or relapsed MM [93]. Responses included 12 patients with stable disease, and one patient maintaining a partial response for greater than 8 months. It was also well tolerated with a good safety profile.

CD137(4-1BB)/CD137L

CD137, also known as 4-1BB, is an induced T-cell costimulator molecule and a member of TNFR superfamily [94]. CD137 is induced on activated CD4 and CD8 T cells, NK cells and constitutively on DCs, Tregs, monocytes and myeloid cells [94,95]. Its ligand 4-1BBL is expressed on B cells, DCs, macrophages, activated T cells and endothelial cells [94–96]. Agonistic CD137 mAb stimulated the proliferation of CD4 and CD8 T cells, mainly CD8 CTL, increased cytokine production, prevent activation induced cell death [94,97] and in absence of cognate signals increases the memory T-cell expansion [98]. Large tumors in mice are eradicated with increased cytotoxic T-cell activity in poorly immunogenic Ag104A sarcoma and highly tumorigenic P815 mastocytoma using agonist CD137 mAb [99]. CD 137 agonist mAbs also increase adhesion molecules ICAM-1, VCAM 1 and E-selectin thus increasing the trafficking of activated T-cells into tumor and also prevent immune tolerance by preventing induction of CD8 CTL anergy to soluble tumor antigens [100].

Urelumab (BMS 663513) is humanized IgG4 mAb and PF 05082566 is humanized IgG2 mAbs for CD137 which are currently in clinical development [101,102]. BMS 663513 was tested in a Phase I/II trial with locally advanced and metastatic solid tumors. Initially patients with melanoma have been

enrolled but enrollment has been expanded to include renal cell and ovarian cancer patients [103]. Treatment is well tolerated and responses included three partial responses and four with stable disease [104]. A randomized Phase II trial (NCT00612664) in metastatic melanoma patients as a second-line therapy was terminated due to grade 4 hepatitis [95]. Urelumab is being tested with rituximab in B-cell non-Hodgkin's lymphoma or chronic lymphocytic leukemia as enhanced antibody-dependent cytotoxicity by rituximab was noted after activation of NK cells with CD137 [105]. Addition of anti-CD137 mAb to cetuximab improves efficiency of cetuximab in head and neck tumors as well as *KRAS* mutant and wild-type colorectal cancer, which provides further evidence for the use of immunotherapy, specifically anti-CD137, in combination with other agents [106]. PF–05082566 was tested in a Phase I study of 27 patients and is well tolerated with mostly grade 1 adverse effects with one grade 3 elevated alkaline phosphatase was noted [107].

GITR

GITR is a member of TNFR superfamily and is a co-stimulatory receptor. It has been originally discovered as up regulated in dexamethasone treated murine T-cell hybridomas [108]. It has very low expression in human T cells but constitutively expressed in human Treg [109]. Upon stimulation, naïve T cells and Treg upregulate GITR in a similar fashion to 4-1BB and OX40 suggesting their role in latter time points rather than early priming [110]. GITR-L is expressed in DCs, macrophages and B cells and is upregulated upon activation. GITR-L is also found in endothelial and activated T cells and may have role in leukocyte adhesion [110]. Its function is similar to OX40 and 4-1BB; it sends costimulatory signals inducing T-cell proliferation, effector function and protects T cells from activation induced cell death [110]. Combined anti-PD-1 blockage and GITR costimulation has potent antitumor activity in murine ID8 ovarian cancer model and is synergistic with chemotherapeutic agents. This combination promotes accumulation of CD4, CD8 T cells with decreased Treg and myeloid derived suppressive cells [111]. TRX518, an anti-GITR mAb, is being studied in a Phase I study in patients with stage III or stage IV melanoma or other solid tumor malignancies (NCT01239134 [86]). To circumvent autoimmune complications from immunomodulators mRNA transfected DCs are used locally to deliver anti CTLA-4 mAb and soluble GITR-L while increasing the antitumor immune responses [112]. Phase I clinical trial of a DC vaccine that entails intranodal injection of DCs transfected with mRNA encoding tumor antigens along with DCs transfected with mRNA encoding soluble human GIRT-L and anti CTLA-4 mAb is in progress (NCT 01216436 [86]).

Tim-3

Tim-3 was first described in 2002 [113]. It is expressed on CD4 T cells and CD8 CTLs. Tim-3 binds several molecules including Gal-9, CEACAM1, HMGB1 as well as glycosylated molecules [114]. Binding of its ligand, Gal-9 induces cell death and thus illustrating its role as a negative regulatory molecule, with particular importance in Th1- and Tc1-driven responses. Tim-3 is expressed on all IFN-γ secreting Th1 cells as well as DCs [113]. Th1 immunity is regulated via binding of its ligand, Gal-9, directly triggering cell death. It has also been shown that Tim-3/Gal-9 binding also suppresses immune responses indirectly by expanding the population of myeloid derived suppressor cells [113]. Tim-3 has been implicated in inducing T-cell exhaustion in several scenarios including chronic viral infections such as hepatitis C and HIV, bacterial infections and cancer [113]. In murine models of colon adenocarcinoma, melanoma and mammary adenocarcinoma, Tim-3 can be co-expressed with PD-1 in tumor infiltrating CD4 and CD8 T cells. Tim-3^{+}PD-1^{+} CD8 T cells were among the most impaired T cells with reduced proliferation and decreased production of IL-2, TNF and IFN-γ. *In vivo*, Tim-3 blockade in concert with PD-1 blockade produced a significantly higher antitumor effect than either one alone. Additionally, this combined blockade increased the frequency of proliferating antigen specific CD8 T cells.

LAG-3

LAG-3, also called CD223, is expressed on activated T cells, NK cells, B cells and tumor infiltrating lymphocytes [115]. It is closely related to CD4; it is a member of the immunoglobulin superfamily and its gene is located near CD4 on chromosome 12. LAG-3 is a negative regulator of T-cell activation and homeostasis [115]. LAG-3 binds to MHC class II molecules with high affinity [116]. LAG-3 cross-linking on activated human T cells induces T-cell functional unresponsiveness and inhibits TCR-induced calcium ion fluxes. Similar to CD4 and CD8, LAG-3 is considered to be a coreceptor to the CD3–TCR complex. Inhibition of cytokines, such as IL-2, is induced by LAG-3 *in vitro*. It decreases the pool of memory CD4 and CD8 T cells. It also increases the suppressive activity of Treg. For maximal suppressive activity of Treg, LAG-3 signaling is required [117]. It was not clear however, if the signal alone was sufficient. Within the tumor microenvironment LAG-3 promotes immune tolerance of the tumor by inhibiting APC and T-cell function [118].

Malignant mouse and human tissue has been shown to co-express PD-L1 and LAG-3 [119]. A significant percentage of tumor infiltrating CD4 and CD8 T cells from mouse tumor models of melanoma, colorectal adenocarcinoma and fibrosarcoma, express high levels of LAG-3 and PD-1. When using anti-LAG-3 immunotherapy, reduced growth of fibrosarcoma and colorectal adenocarcinoma is observed in some mice. This same effect is seen with anti-PD-1 monotherapy. Anti-LAG-3 produced a synergistic effect when combined with anti-PD-1 immunotherapy with 70% of the fibrosarcoma and 80% of colorectal adenocarcinoma mice noted to be tumor free [119]. This regimen, however, was shown to have no effect on the melanoma model. Interestingly, treating mice with anti-LAG-3/anti-PD-1 combined therapy is proposed to be less toxic given that LAG-3 and PD-1 co-expression is largely limited to tumor infiltrating lymphocytes.

IMP321 is a clinical grade LAG-3-Ig recombinant fusion protein that antagonizes normal LAG-3 functioning [120]. In 2009 the results of the first Phase I study involving IMP321 alone was released. Patients with advanced renal cell carcinoma were treated [121]. No significant adverse events occurred. Tumor growth reduction was seen and progression-free survival was better in those patients receiving higher doses (>6 mg). Out of eight patients treated with high dose IMP321, seven had stable disease at 3 months compared with only three of 11 in the lower dose group. Another Phase I trial of IMP321 and paclitaxel in metastatic breast cancer was conducted [122]. Patients treated with IMP321 were found to have a sustained increase in the number and activation of monocytes and DCs as well as an increase in the percentage of NK and long lived cytotoxic effector memory CD8 T cells, which correlates well with the preclinical data. Additionally 90% of patients had some clinical benefit, with only three of 12 patients progressing by 6 months. Objective tumor response rate was 50% compared with the historic control group of 25% [122]. IMP321 is well tolerated as well. There were several other Phase I trials combining IMP321 with other agents. In 18 patients with advanced pancreatic adenocarcinoma, IMP321 was combined with conventional gemcitabine and had a good safety profile [123]. Its clinical benefit was hard to evaluate likely due to suboptimal dosing of gemcitabine. IMP321 was combined with an anticancer vaccine MART-1 peptide and used in 12 patients with advanced melanoma [124]. Six patients received MART-1 alone and six in combination with IMP321. One patient experienced a partial response in the IMP321 group and none in the MART-1 alone group.

BTLA

BTLA (CD272) is a transmembrane protein that is expressed on Th1 cells as well as B cells and DCs [125–127]. BTLA, via interaction with HVEM (herpes virus entry mediator), inhibits cancer specific CD8 T cells [127]. HVEM is expressed in hematopoietic cells, including B and T cells, as well as in nonhematopoietic cells (parenchymal cells) [126]. HVEM is also expressed in melanoma cells and variety of solid tumors [126]. Hodgkin's lymphoma, B-cell non-Hodgkin's lymphoma and some T-cell non-Hodgkin's lymphomas use BTLA for immune evasion [127]. BTLA and HVEM are highly expressed in B-cell chronic lymphocytic leukemia suggesting their role in pathogenesis [126,128]. BTLA–HVEM is implicated in Vγ9Vδ2 T-cell proliferation, differentiation and has a critical role in their control of lymphogenesis [129]. T-cell responses against minor histocompatibility antigens on malignant cells play an important role for cure in hematological malignancies after allogeneic stem cell transplant via graft versus tumor effect. BTLA suppresses minor histocompatibility antigen-specific CD8 T cells after allogeneic stem cell transplant thus providing a rationale for its clinical utility in post transplantation therapies [130]. High BTLA expression is associated with shorter survival in gastric cancer [131]. BTLA expression is upregulated on cytotoxic CD8 T cells in peripheral blood of patients with hepatocellular carcinoma and correlates with disease progression and increased expression is associated with high recurrence rates [132]. Downregulation of BTLA *in vivo* can be attained by adding CpG oligonucleotides to vaccine formulation, which leads to increased resistance to BTLA/HVEM inhibition [133]. Because of the role of BTLA/HVEM in hematological as well as solid tumors, BTLA inhibition also represents a target for cancer immunotherapy.

IDO synthase

Tryptophan plays an important role in peripheral immune tolerance through its rate limiting tryptophan degradation along the kynurenine pathway, including IDO1 and tryptophan-2,3-dioxygenase (TDO). Shortage of tryptophan leads to cell cycle arrest, decreased proliferation through inactivation of mTOR pathway, while tryptophan metabolites can cause T-cell apoptosis, and induce differentiation of Tregs. Tumors can evade the immune system by hijacking tryptophan catabolizing enzymes IDO1 and TDO [134]. IDO1 protein is expressed in mature DCs in lymphoid tissues, some epithelial cells of female genital tract, placental and pulmonary endothelial cells [135]. IDO1 positive cells are scattered in human and tumoral lymphoid tissues particularly in cervical, colorectal and gastric carcinomas. It is highly present in vascular cells

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD27						
Varlilumab (CDX-1127)	I	Dose escalation (0.1–10 mg/kg)	170	B-cell malignancies and solid tumors	December 2015	NCT01460134
	I	Stereotactic body radiation therapy randomized to before, after or with immunotherapy	21	Castrate resistant prostate cancer	March 2017	NCT02284971
	Ib	Two dose levels in combination with vaccine ONT-10	42	Breast and ovarian cancer	May 2016	NCT02270372
	I/II	Dose escalation of varlilumab with nivolumab	190	Advanced solid tumors	December 2017	NCT02335918
	I/II	Dose escalation of varlilumab with sunitinib	58	Metastatic clear cell carcinoma	March 2019	NCT02386111
	I/II	Dose escalation of varlilumab with ipilimumab	100	Unresectable stage III or IV melanoma	December 2020	NCT02413827
OX40 & OX40L						
MEDI6469	Ib	Dose escalation study of drug given at different time intervals before surgery	55	Advanced head and neck squamous cancer	October 2019	NCT02274155
	I/II	In combination with radiation therapy and escalating doses of cyclophosphamide	37	Metastatic prostate cancer	October 2016	NCT01303705
	Ib/II	Alone or in combination with tremelimumab, MEDI4736 or rituximab	212	Advanced solid tumors or diffuse large B-cell lymphoma	October 2017	NCT02205333
MEDI0562	I	First in human	50	Selected solid tumors	November 2019	NCT02318394
MEDI6383	I	Alone or in combination with MEDI4736	212	Advanced solid tumors	April 2019	NCT02221960
Anti OX40 mab	I/II	Stereotactic body radiation therapy to metastatic lesions with anti OX49 antibody	40	Breast cancer patients with liver or lung metastasis	February 2023	NCT01862900
Anti OX40 mab 9B12	I	Dose escalation study	30	Advanced cancer	January 2015	NCT01644968
CD40 & CD40L						
Dacetuzumab (SGN-40)	I	Dose escalation	50	Relapsed or refractory non-Hodgkin's lymphoma	March 2007	NCT00103779
	I	Dose escalation	44	Recurrent or refractory multiple myeloma	November 2007	NCT00079716
	I	Dose escalation in combination with lenalidomide and dexamethasone	36	Relapsed multiple myeloma	February 2010	NCT00525447
	Ib	In combination with bortezomib	18	Multiple myeloma	April 2010	NCT00664898

Data taken from [86].

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD40 & CD40L (cont.)						
	Ib	In combination with rituximab	22	Follicular and marginal zone lymphoma	November 2010	NCT00556699
	Ib	In combination with rituximab and gemcitabine	33	Diffuse large B-cell lymphoma	February 2010	NCT00655837
	II	Monotherapy	46	Relapsed diffuse large B-cell lymphoma	January 2009	NCT00435916
	I/II	Dose escalation	12	Chronic lymphocytic leukemia	October 2006	NCT00283101
CP-870,893	I	Dose escalation in combination with tremelimumab	32	Metastatic malignant melanoma	October 2014	NCT01103635
	I	Dose escalation study in combination with gemcitabine	22	Unresectable pancreatic cancer	January 2011	NCT00711191
	I	Dose escalation	29	Advanced solid tumors	February 2006	NCT02225002
	I	Dose escalation in combination with carboplatin and paclitaxel	34	Metastatic solid tumors	July 2009	NCT00607048
Lucatumumab (HCD122)	I	Dose escalation	28	Multiple myeloma	May 2009	NCT00231166
	1b	In combination with bendamustine	?	Follicular lymphoma	May 2012	NCT01275209
	Ia/II	Dose escalation	111	Lymphomas	February 2013	NCT00670592
SEA-CD40	I	Dose escalation	144	Advanced solid tumors	March 2019	NCT02376699
APX005M	I	Dose escalation	32	Advanced solid tumors	December 2017	NCT02482168
Recombinant CD40L	I	Dose escalation in combination with recombinant FLT3 ligand	?	Metastatic melanoma or kidney cancer	Completed	NCT00020540
Chi Lob 7/4	I	Dose escalation	29	Advanced cancer	October 2014	NCT01561911
ADC–1013	I	Dose escalation	40	Advanced solid tumors	October 2017	NCT02379741
AdCD40L	I/IIa	Alone or in combination with low dose cyclophosphamide with or without radiation	30	Advanced cancer	December 2017	NCT01455259
RO7009789	Ib	Dose escalation in combination with MPDL3280A	160	Advanced solid tumors	December 2017	NCT02304393

Data taken from [86].

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD137 & CD137L						
Urelumab (BMS663513)	I	Dose escalation	122	Advanced cancers	March 2019	NCT01471210
	Ib	In combination with cetuximab	104	Advanced colorectal carcinoma or head and neck squamous cell carcinoma	January 2017	NCT02110082
	Ib	Dose escalation in combination with rituximab	74	B-cell non-Hodgkin's lymphoma	December 2018	NCT01775631
	I/II	Dose escalation in combination with nivolumab	200	Advanced cancers	December 2018	NCT02253992
	II	In combination with rituximab	24	Advanced chronic lymphocytic leukemia	July 2020	NCT02420938
PF-05082566	I	Dose escalation and in combination with rituximab in B-cell lymphomas	126	Advanced solid tumors or B-cell non-Hodgkin's lymphoma	December 2017	NCT01307267
	Ib	Dose escalation in combination with mogamulizumab	70	Advanced cancers	June 2018	NCT02444793
	Ib	Dose escalation in combination with pembrolizumab	45	Advanced solid tumors	May 2017	NCT02179918
GITR						
TRX518	I	Dose escalation	40	Advanced solid tumors	December 2015	NCT01239134
LAG-3						
IMP321	I	Dose escalation	24	Advanced renal cell carcinoma	October 2008	NCT00351949
	I	Dose escalation in combination with paclitaxel	33	Metastatic breast cancer	January 2010	NCT00349934
	I	Dose escalation in combination with gemcitabine	18	Advanced pancreatic cancer	September 2012	NCT00732082
BMS-986016	I	Dose escalation in combination with nivolumab	198	Advanced solid tumors	May 2018	NCT01968109
	I	Dose escalation	88	Chronic lymphocytic leukemia, lymphomas and multiple myeloma	June 2018	NCT02061761
LAG525	I/II	Dose escalation in combination with PDR001	240	Advanced solid tumors	November 2017	NCT02460224

Data taken from [86].

in renal cell cancer [135]. Expression of IDO1 is highest in endometrial and cervical cancers followed by kidney and lung [135]. IDO1 expression is associated with aggressive phenotype, poor prognosis, shorter survival and increased Tregs [136]. IDO1 inhibitors were shown to exhibit antitumor effect in mouse models alone as well as have synergistic effects with a variety of chemotherapeutic agents in preclinical models [137–140]. Indoximod is an IDO inhibitor that is being studied in a Phase II trial with taxane chemotherapy in metastatic breast cancer (NCT 01792050 [86]). IDO inhibitors can provide a synergistic effect when administered with vaccines and immunotherapy; IDO induction in response to inflammation can attenuate antitumor vaccine [139]. This provided the rationale for a randomized Phase II study of Indoximod with Sipuleucel-T (Provenge®) in the treatment of patients with asymptomatic or minimally symptomatic metastatic castration resistant prostate cancer (NCT01560923 [86]). Other studies include a Phase I study of NLG–919, an IDO inhibitor, for patients with advanced solid tumor malignancies and a Phase I/II trial of the indoximod in combination with ipilimumab for the treatment of unresectable advanced stage melanoma [141,142].

Conclusion & future perspective

Our understanding of immune dysfunction in cancer continues to develop. Growth and progression of cancer are made possible by the ability of the malignant cells to manipulate immune checkpoint pathways that prevent immune overstimulation. Significant progress has been made in the field with mAb against CTLA-4 and now PD-1 in clinical use. This gives credence to the efforts aimed at developing agents targeting other immune modulatory pathways. The success PD-1 inhibitors may very well translate to PD-L1 inhibitors being successful in the clinic. PD-L1 is widely expressed in a variety of tumors and PD-L1 inhibitors have shown impressive, albeit preliminary, results in areas of unmet need such as urothelial bladder cancer which has been resistant to conventional chemotherapy [34,50]. Other B7 family members are emerging as clinical target in preclinical models such as B7x, HHLA2 and B7-H3. Other immunoglobulin superfamily members such as Tim-3 and VISTA also show promise in preclinical models. LAG-3 targeting molecules have also reached clinical trials with good safety profile and evidence of antitumor efficacy.

TNFR superfamily member also hold promise in cancer immunotherapy. Preclinical and clinical data support targeting CD27 where a durable response was noted as well [81,82]. mAb targeting CD40L have reached clinical trials although the clinical experience in the CD40/CD40L pathway illustrates some of the caution needed with immune disinhibition, particularly in unwanted side effects such as cytokine release syndrome [88,92,93]. OX40, CD137 and GITR antibodies have also reached clinical trials providing further evidence that TNFR family members are viable targets for cancer immunotherapy [101–104,112]. Table 1 is the summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

The redundancy in inhibitory immune checkpoint molecules sheds light on the complexity of immune regulation. It also allows for the targeting of these molecules in succession or in combination. A new pathway may be targeted after the efficacy of an earlier immunotherapy is exhausted as evidenced by the success of PD-1 targeting mAbs in ipilimumab-refractory tumors indicating that tumors may recruit additional pathways when one is blocked, or several pathways may be in fact be recruited simultaneously by tumor cells [24,25,31]. Efficacy of ipilimumab in combination with nivolumab also illustrates that tumors may recruit more than one inhibitory pathway at the same time [143].

Another interesting dilemma that emerges as cancer immunotherapy gains momentum is integrating these novel agents with current regimens that mainly consist of conventional cytotoxic chemotherapy. On one hand, conventional wisdom may lead us to believe that chemotherapy may be synergistic. The immunosuppressive effect of chemotherapy however can have unpredictable effects on the immune system. Cyclophosphamide has been used as a Treg depleting agents in preclinical models with some success [144,145]. Combination regimens have also reached clinical trials [34,122,123]. Combination of immune checkpoint inhibitors have also been studied with traditional immunotherapies or targeted therapies such as rituximab and cetuximab where they show efficacy and tolerability [105,106,146]. With evidence pointing to better outcomes in second line setting or even first line setting of immunotherapies, cancer immunotherapy will play a vital role in the future of oncology [24,31,147].

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Executive summary

Cancer immunotherapy targets in clinical trials

- PD L-1 blockade can restore cytotoxic T-lymphocyte antitumor activity.
- CD27 is a hematologic malignancy marker that has been targeted in Phase I trials.
- Lag-3 is a co-inhibitory molecule and blocking antibodies have reached clinical trials.
- CD40, CD137 and GITR agonism may play a role in cancer immunotherapy and have reached Phase I and Phase II trials.
- Indoleamine 2,3-dioxygenase is not a cell-surface protein and inhibitors have reached clinical trials.

Emerging targets for cancer immunotherapy still in preclinical study

- B7x, HHLA 2, B7-H3 are B7 family members that have inhibitory role and are expressed by tumor cells.
- Tim-3, VISTA and BTLA blockade are emerging targets in preclinical models.
- Agonism of stimulatory molecules OX40 and OX40L may also play a role in cancer immunotherapy.

Conclusion

- T-cell disinhibition is now a clinically effective approach.
- Targeting of several pathways may be done in succession or in concert.
- Immunotherapy can be more effective than chemotherapy especially in the second-line setting.

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Clinical Cancer Research

Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy

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Abstract

B7-H3 (CD276) is an important immune checkpoint member of the B7 and CD28 families. Induced on antigen-presenting cells, B7-H3 plays an important role in the inhibition of T-cell function. Importantly, B7-H3 is highly overexpressed on a wide range of human solid cancers and often correlates with both negative prognosis and poor clinical outcome in patients. Challenges remain to identify the receptor(s) of B7-H3 and thus better elucidate the role of the B7-H3 pathway in immune responses and tumor evasion. With a preferential expression on tumor cells, B7-H3 is an attractive target for cancer immunotherapy. Based on the clinical success of inhibitory immune checkpoint blockade (CTLA-4, PD-1, and PD-L1), mAbs against B7-H3 appear to be a promising therapeutic strategy worthy of development. An unconventional mAb against B7-H3 with antibody-dependent cell-mediated cytotoxicity is currently being evaluated in a phase I clinical trial and has shown encouraging preliminary results. Additional therapeutic approaches in targeting B7-H3, such as blocking mAbs, bispecific mAbs, chimeric antigen receptor T cells, small-molecule inhibitors, and combination therapies, should be evaluated, as these technologies have already shown positive results in various cancer settings. A better understanding of the B7-H3 pathway in humans will surely help to further optimize associated cancer immunotherapies. *Clin Cancer Res; 22(14); 3425–31. ©2016 AACR.*

Background

During an immune response, naïve T cells engage their T-cell receptor (TCR) to interact with a complex of MHC and peptide expressed by antigen-presenting cells (APC). This first signal is not sufficient to trigger full T-cell activation. A second signal is provided by the interaction of costimulatory molecules (most importantly B7-1/2 and CD28), leading to full T-cell activation. Following activation, coinhibitory molecules, such as CTL-associated protein 4 (CTLA-4), function to restrain T-cell responses, resulting in T-cell exhaustion and tolerance. Interactions between members of the B7 ligand family and the CD28 receptor family provide T-cell costimulation and coinhibition, regulating T-cell activation and tolerance, exhaustion and effector function, differentiation, and memory generation. B7-H3, also known as CD276, is an immune checkpoint molecule belonging to the B7-CD28 pathways.

Structure and functional significance of the B7-H3 pathway

B7-H3 is a type I transmembrane protein encoded by chromosome 9 in mice and 15 in humans. The extracellular domain is composed of a single pair of immunoglobulin variable domain and immunoglobulin constant domain in mice (2IgB7-H3 isoform) and two identical pairs in human (4IgB7-H3 isoform) due to exon duplication (1, 2). The intracellular tail of B7-H3 is short and has no known signaling motif. B7-H3 was first described in humans (3) and then in mice (2) but is universally expressed among species (4). A soluble form, cleaved from the surface by a matrix metallopeptidase (MMP; ref. 5) or produced through alternative splicing of the intron (6), is also detectable in human sera.

B7-H3 is expressed on many tissues and cell types. At the mRNA level, it is ubiquitously found in such nonlymphoid and lymphoid organs as the liver, heart, prostate, spleen, and thymus. Despite broad mRNA expression, protein expression is limited at steady state, suggesting the presence of an important posttranscriptional control mechanism. B7-H3 is constitutively found on nonimmune resting fibroblasts, endothelial cells (EC), osteoblasts, and amniotic fluid stem cells. Moreover, B7-H3 expression is induced on immune cells, specifically APCs. In particular, coculture with regulatory T cells (7), IFNγ, lipopolysaccharide (LPS), or anti-CD40 *in vitro* stimulation (8) all induce the expression of B7-H3 on dendritic cells (DC). Monocytes and monocyte-derived DCs upregulate B7-H3 after LPS stimulation or cytokine-induced differentiation, respectively (9). In addition, B7-H3 is also detected on natural killer (NK) cells, B cells, and a minor population of T cells following PMA/ionomycin stimulation (1).

The B7-H3 pathway has a dual role in contributing to the regulation of innate immune responses. One study found that neuroblastoma cells express B7-H3 on their cell surface, which protect them from NK cell–mediated lysis (10). Another group argues that B7-H3 costimulates innate immunity by augmenting proinflammatory cytokines release from LPS-stimulated monocytes/macrophages, in both a Toll-like receptor 4- and 2–dependent manner (11). The role of B7-H3 in controlling the innate immunity is clearly complex and requires more elucidation.

A larger body of literature suggests that B7-H3 plays an important role in T cell–mediated adaptive immunity, although the nature of its signalling remains controversial (12). A

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costimulatory role of B7-H3 on human T cells was initially reported *in vitro* (3). Murine studies showing B7-H3 worsens experimental autoimmune encephalomyelitis (EAE), arthritis, bacterial meningitis, and chronic allograft rejection (13–15) supported this claim. However, subsequent studies have mostly shown that B7-H3 acts as a T-cell coinhibitor. B7-H3 inhibits polyclonal or allogeneic CD4 and CD8 T-cell activation, proliferation, and effector cytokine production (IFNγ and IL2) in mice and humans. This negative regulation of T cells is associated with diminished NFAT, NF-κB, and AP-1 transcriptional factor activity (16). Researchers from independent studies using either protein blockade or gene-knockout mice have reported that B7-H3 ameliorates graft-versus-host disease, prolongs cardiac allograft survival, reduces airway hypersensitivity, and delays EAE onset, especially by downregulating Th1 responses (8, 17, 18). These examples lend more credence to the coinhibitory nature of B7-H3.

The receptor(s) for B7-H3 has yet to be discovered (19, 20). Nevertheless, the crystal structure of mouse B7-H3 reveals that its receptor engagement on T cells involves the particular segment connecting F and G strands (the FG loop) of the immunoglobulin variable domain of B7-H3 (19). Moreover, B7-H3 crystallizes as a glycosylated monomer but also undergoes an unusual dimerization *in vitro*. Together, the nature of the receptor(s), differences in cellular context, and various disease models certainly account for the discrepancies in the function of the B7-H3 pathway in regulating both innate and adaptive immunity during homeostasis and inflammation.

Beyond the immune system, the B7-H3 pathway has a nonimmunologic role in promoting osteoblastic differentiation and bone mineralization in mice, ensuring normal bone formation (21). Indeed, B7-H3 knockout mice had reduced bone mineral density and were more susceptible to bone fractures compared with wild-type mice. Furthermore, similar to other immune checkpoints of the B7-CD28 pathways, B7-H3 is also expressed in human cancers and participates in tumorigenesis through modulation of both immune and non–immune-related pathways.

B7-H3 in the tumor microenvironment and immune evasion

Numerous studies have described B7-H3 overexpression in human malignancies, including melanoma (22), leukemia (23), breast cancer (24), prostate cancer (25), ovarian cancer (26), pancreatic cancer (27), colorectal cancer (28), and other cancers. As detected by immunochemistry technique, more than 60% and up to 93% of patient tumor tissues display aberrant expression of B7-H3 in the vast majority of cancer types (Table 1), while limited expression is seen on normal healthy tissues. Within positively stained samples, B7-H3 is found on the membrane, in the cytoplasm, or within the nucleus of cancer cells but also on the tumor-associated vasculature. In a study of more than 700 colorectal cancer patients, cytoplasmic/membrane and stromal expression were respectively seen in 86% and 77% of the samples, whereas nuclear expression of B7-H3 in cancer cells was present in 27% of the samples (29). In most studies, the intensity of the B7-H3 staining was further quantified and ranged from low to high expression. Finally, association studies investigated potential clinical correlation between tumor-associated B7-H3 and disease severity. Various clinicopathologic parameters were assessed, including tumor size, metastasis, cancer stage, survival, and recurrence rate. In most cases, a high expression of B7-H3 was correlated with bad prognosis and poor clinical outcome. One study with more than 800 prostate cancer patients revealed that patients with strong B7-H3 expression on tumor cells had a significantly increased risk of disease spread at the time of surgery, clinical cancer recurrence, and cancer-specific death (25). B7-H3 expression in lung cancer was associated with a lower number of tumor-infiltrating lymphocytes and with lymph node metastasis, suggesting a role for B7-H3 in immune evasion and tumorigenesis (30). Importantly, B7-H3 protein expression in tumors is known to be modulated by miR-29 (31), upregulated upon IFNγ stimulation (32), and potentially increased by immunoglobulin-like transcript 4 signaling (33).

To date, the molecular mechanisms by which B7-H3 participates in tumor growth and immune evasion still remain elusive and need further investigation. Interestingly, aberrant glycosylation of B7-H3 was described in oral cancer. Its glycans, more diverse and with higher fucosylation, seem to interact better with DC-SIGN [DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin] and Langerin (34), proteins expressed on the membrane of DCs, suggesting a possible engagement and tolerization of DCs. Moreover, the cross-talk between lung cancer cells and tumor-associated macrophages, partially through IL10, induces B7-H3 membrane expression and inhibits T-cell antitumor immunity in mice (35). Besides its role in modulating tumor immunity, B7-H3 also has a nonimmunologic function in regulating tumor aggressiveness. It was shown to modulate migration, invasion, and adhesion to fibronectin of various cancer cells (36) through the Jak2/Stat3/MMP-9 signaling pathway (37). In addition, overexpression of B7-H3 in colorectal and breast cancer cells augments resistance to apoptosis by activating the Jak2/STAT3/survivin signaling pathway. This, in turn, weakens tumor cell sensitivity to the chemotherapeutic drug paclitaxel (38, 39). Furthermore, B7-H3 was shown to modulate the metastasis-associated proteins MMP-2, TIMP-1, TIMP-2, STAT3, and IL8 in melanoma cells (40). In hepatoma cells, B7-H3 targeted the epithelial-to-mesenchymal transition via the Jak2/STAT3/Slug signaling pathway (41). Finally, a recent study showed that decreased expression of B7-H3 reduces the glycolytic capacity and sensitizes breast cancer cells to AKT/mTOR inhibitors, unveiling a previously unknown link between B7-H3 and metabolism (42). Together, these mechanisms promote aggression and invasion of the tumor.

Clinical–Translational Advances

The precise role of B7-H3 in regulating the function of tumor-infiltrating immune cells and its activity in cancer cells has yet to be fully elucidated. This absence is due in large part to the conflicting studies that have demonstrated B7-H3 to be either costimulatory or coinhibitory in several disease models. In addition, the receptor(s) that interact with B7-H3 have yet to be identified, magnifying the scrutiny. However, there is no doubt that aberrant expression of B7-H3 consists of a possible biomarker and a promising immune checkpoint target for multiple cancer immunotherapy approaches (Fig. 1), as anticipated almost 10 years ago (43). The scientific community is beginning to explore its therapeutic role in cancer in a variety of ways.

Blocking mAbs

The B7 ligand and CD28 receptor families have become attractive targets for cancer immunotherapy, with specific emphasis placed on the development of mAb blocking B7-CD28 pathways. Blocking mAbs against the immune checkpoints CTLA-4,

Table 1. B7-H3 aberrant expression in human cancers and association with clinical–pathologic characteristics

Cancer type	B7-H3–expressing tumor tissues	Clinical correlation	References
Hepatocellular carcinoma	93.8 %	Poorer survival, increased recurrence	(32)
Pancreatic cancer	93.7%	Lymph node metastasis, lower differentiation grade	(27)
Prostate cancer	- 93%	- Disease spread, increased risk of clinical cancer recurrence, and cancer-specific death	(25)
	- 100%	- Larger tumor volume, extraprostatic extension, higher Gleason score, seminal vesicle involvement, positive surgical margins, >4-fold increased risk of cancer progression after surgery	(62)
Osteosarcoma	91.8%	Shorter survival and recurrence time, lower CD8 TIL	(63)
Breast cancer	- 90.60%	- Lymph node metastasis, advanced disease, IL10 in tumor cells	(64)
	- 80.55%	- Negative relation with VEGF, microvascular density for CD34, and tumor size	(24)
Colorectal cancer	- Cytoplasmic/membrane 86% Stroma 77% Nuclear 27%	- Reduced recurrence-free survival in TNM stage I	(28)
	- Cytoplasm 62% Membrane 46% Nuclear 30%	- Reduced metastasis-free, disease-specific, and overall survival	(29)
Ovarian carcinoma	Cytoplasm/membrane 83% Tumor endothelium 44%	High-grade serous histologic subtype, increased recurrence, and reduced survival	(26)
Endometrial cancer	75.7%	TIL infiltration, shortened overall survival	(65)
Oral squamous cell carcinoma	74.75%	Larger tumor size, advanced clinical stage, low survival rate	(34)
Cervical cancer	72.22%	Tumor size, positive correlation with FoxP3, negative correlation with IL2	(66)
Non–small cell lung cancer	- 69.5%	- Lymph node metastasis, TNM stage	(67)
	- 37.1%	- Lower TILs, lymph node metastasis	(30)
Bladder cancer	58.6%	No association	(68)
Clear cell renal cell carcinoma	Cancer cells 19% Tumor vasculature 18%	Large tumor size, advanced TNM stage, high nuclear grade, coagulative tumor necrosis, and capsular invasion	(69)
Glioma	Not specified	Malignancy grade	(70)
Melanoma	Not specified	Stage of melanoma, melanoma-specific survival in stages III and IV	(22)

NOTE: Not all clinical studies were included in this table due to the space limitation.
Abbreviations: TIL, tumor-infiltrating lymphocyte; TNM, tumor node metastasis classification.

programmed cell death protein 1 (PD-1), and PD-1 ligand 1 (PD-L1) have shown significant clinical success in patients with a variety of cancers (44–46). This same logic and success can be extended to B7-H3 as well (Fig. 1A). Blocking mAbs are effective because they either partially or completely neutralize inhibitory ligand-to-receptor interactions, thus allowing effector functions. Despite the fact that the B7-H3 binding partner(s) remains unknown and that mAbs generated against B7-H3 are specific to the protein, the ability of these mAbs to neutralize B7-H3 interactions and the signaling pathway remains unknown. Thus, currently, no blocking mAb against B7-H3 is available. Until this receptor (or receptors) is found, additional strategies in screening antibodies for neutralization capacity need to be developed.

Targeting B7-H3 through antibody-dependent cell-mediated cytotoxicity and antibody–drug conjugate therapies

The difficulties that have been encountered in creating blocking mAbs against B7-H3 have led to the optimization of antibodies against B7-H3 for therapy through alternative means (Fig. 1B). Enoblituzumab (MGA271), a mAb reactive to cancer-associated B7-H3 showed enhanced antitumor function through potent antibody-dependent cell-mediated cytotoxicity (ADCC) against a broad range of tumor cell types. In mice, weekly doses of MGA271 in both renal and bladder carcinoma xenografts resulted in sustained tumor growth inhibition, effects that were Fc mediated (47). Currently, an ongoing phase I study of enoblituzumab is being conducted in patients with refractory B7-H3–expressing tumors or B7-H3–expressing vasculature (trial NCT01391143). Preliminary results of the dose-escalation study indicate that as a monotherapy, the Fc-enhanced mAb enoblituzumab shows antitumor activity in several tumor types and modulates T cells by increasing the T-cell repertoire clonality in the peripheral blood of patients following treatment (48). Although enoblituzumab is not a blocking mAb and its success largely depends on ADCC, the results are encouraging and open the door for more clinical trials targeting this protein by way of mAbs.

Alternatively, mAbs can be stably conjugated to a biologically active cytotoxic drug or compound that induces cell death. Once the mAb binds the cell-surface antigen, the complex is internalized, releasing the cytotoxic substance and killing cancer cells. 8H9 is a mAb specific to B7-H3 that showed clinical success as an antibody–drug conjugate (ADC) after it was radiolabeled to iodine-131 (^{131}I) and administrated to patients with metastatic central nervous system (CNS) neuroblastoma (49). 8H9 also distinguishes itself from other B7-H3–specific antibodies in that it binds to the FG loop of B7-H3, a region critical to its immunologic function (50). Recently, 8H9 was humanized and affinity matured and maintained its ability to kill B7-H3–positive neuroblastoma cells *in vitro*. Two-fold and 5-fold enhancements in

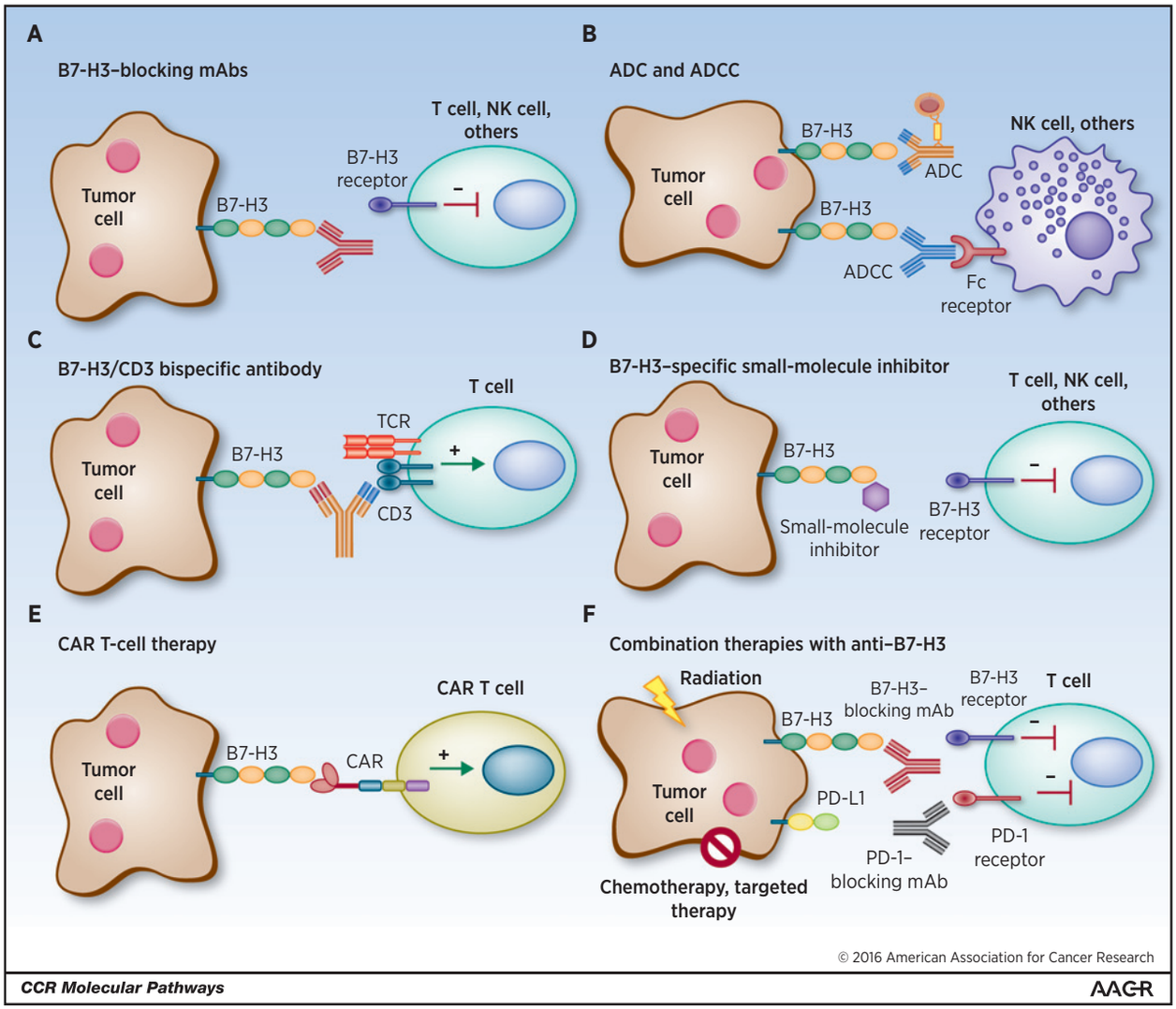

Figure 1.
Human cancer immunotherapy strategies targeting B7-H3 A, blockade of B7-H3 with blocking mAbs neutralizes inhibitory signaling in its unidentified receptor(s) in T cells, NK cells, and other immune cells enabling effector function. B, B7-H3–specific ADCC initiated by Fc receptor engagement of NK cells and other immune cells induces death of tumor cells. ADCs bind to B7-H3 expressed by tumor cells and are internalized and generate cytotoxicity to tumor cells. C, CD3/B7-H3–bispecific antibodies bind to tumor-expressed B7-H3 and crosslink the CD3 portion of the TCR complex, activating T cells in the tumor microenvironment for tumor cell death. D, small-molecule inhibitors may bind to specific regions of B7-H3, such as the FG loop of the IgV domain, inhibiting the ligand–receptor interaction between tumor cells and immune cells, thus blocking receptor signaling and restoring effector function of immune cells. E, engineered CAR T cells recognize membrane B7-H3 and directly kill tumor cells. F, blocking mAbs against B7-H3 in combination with radiation, chemotherapy, targeted therapy, or other immune checkpoint inhibitors synergize to generate more effective antitumor immune responses.

killing were observed in the affinity-matured and humanized 8H9 compared with the nonmatured and chimeric generations, respectively. Furthermore, the mAb was labeled with ^{131}I and injected into athymic nude mice xenografted with human neuroblastoma and showed successful biodistribution to the tumor (50). Currently, clinical trials with radiolabeled 8H9 are ongoing in patients with peritoneal cancers, gliomas, and advanced CNS cancers (NCT01099644, NCT01502917, and NCT00089245).

Bispecific antibodies

Bispecific antibodies are another suitable option beginning to pick up steam in the area of tumor immunotherapy. Bispecific antibodies are artificially generated antibodies composed of fragments of two distinct mAbs, thus combining two specificities. One arm can bind to the CD3 component of the TCR complex on T cells, while the other arm recognizes a tumor-specific antigen, for instance B7-H3, overexpressed on cancer cells (Fig. 1C). That way, T cells are recruited to the tumor site and activated to kill cancer cells (51). Given the upregulated expression of B7-H3 on multiple cancers, it seems like a promising option that should be pursued. Side effects of bispecific antibody treatment include an excessive inflammatory reaction due to cytokines produced by overactivated T cells but can be limited by corticosteroid administration.

Targeting B7-H3 with small-molecule inhibitors

With no current information known about the receptor(s) of B7-H3, the only viable target for disruption of this pathway is tumor-expressed B7-H3. In addition to conventional therapeutic mAbs, the roles of small-molecule inhibitors have also begun to gain interest in the immune-oncology field (52). Small-molecule inhibitors are low-molecular-weight organic compounds (dinucleotides, peptides, monosaccharides, etc.) that bind specific biological targets. They are readily used because of the advantages they offer in cheaper manufacturing costs, ease of delivery due to oral administration, greater tissue distribution due to size, and shorter half-life when compared with antibodies. Knowing that the receptor(s) of B7-H3 on activated T cells engages the FG loop of the IgV domain of B7-H3 (19), a small-molecule inhibitor could be designed to disturb this specific ligation area (Fig. 1D). Although often unpredictable, off-target effects can arise and should be assessed as thoroughly as possible to limit detrimental consequences.

Targeting B7-H3 with chimeric antigen receptor T cells

Another interesting way to target B7-H3 for immunotherapy is with chimeric antigen receptor (CAR) T-cell technology (Fig. 1E). This therapy recently had outstanding results in treating human refractive acute lymphoblastic leukemia (53, 54). Autologous T cells are engineered with a CAR targeting a tumor antigen and adoptively transferred to patients to kill cancer cells. So far, this technology has been successfully applied to hematologic cancers only. Although this area of research is challenging, efforts are being made to translate CAR T-cell therapy to the treatment of solid tumors. Importantly, the target must be highly overexpressed by the tumor and low or absent in normal peripheral tissues, as B7-H3, to avoid off-tumor effects. Engineered T cells would have to reach the tumor site and penetrate the stroma to specifically kill the targeted tumor cells. Moreover, CAR T cells would be exposed to the immunosuppressive tumor microenvironment, which could alter their function. Some optimizations of CAR T cells are currently being made and will hopefully help, either alone or in a combination therapy, to treat solid cancers (55). One clinical trial has evaluated the safety and antitumor activity of CAR T cells in patients with chemotherapy-refractory metastatic pancreatic cancer, with preliminary evidence of good tolerance and antitumor efficacy (56). Of note, a cytokine release syndrome has been described in some patients and must be addressed to fully ensure the safety of this technique.

Synergistic options with anti–B7-H3 therapy: Chemotherapy or targeted therapy, immune checkpoint inhibitors, and radiation

The clinical successes of mAbs blocking immune checkpoints, such as CTLA-4, PD-1, and PD-L1, have led to the rationale of combining these modalities with conventional therapeutics or additional checkpoint inhibitors, with the goal of synergizing their actions and improving patient survival. The most traditional therapeutic regimen for treating cancers has been with chemotherapy. Recent studies have shown that the combination of a variety of chemotherapeutics with checkpoint inhibitors displays great synergistic effects that enhanced the prospects of its full utilization in standard clinical practice. The combination of an anti–CTLA-4 mAb (ipilimumab) and the chemotherapeutic drug dacarbazine, when compared with dacarbazine plus placebo, led to improved overall survival in patients with metastatic melanoma (57). On the basis of a few preclinical animal studies, the combination of B7-H3 blockade and chemotherapy looks promising (Fig. 1F). Indeed, the silencing of B7-H3 through shRNA in an histiocytic lymphoma-derived human cell line, U937, in combination with the antineoplastic drug Ara-C, led to 80% tumor reduction compared with the 40% inhibition observed in wild-type U937 cells combined with Ara-C in a mouse xenograft model (58). Similarly, shRNA silencing of B7-H3 in a murine model of breast cancer, combined with the chemotherapeutic paclitaxel, led to an approximately 80% reduction in tumor growth compared with the untreated wild-type cells (38). In both studies, silencing B7-H3 significantly enhanced tumor cell chemosensitivity and drug-induced apoptosis. Moreover, exploiting the differences between normal cells and cancer cells through targeted therapy as opposed to conventional chemotherapy may also deliver exciting results as a combination strategy. Taken together, these studies provide a rationale for the potential synergistic effects between B7-H3 blockade and chemotherapy or targeted therapy for patients with a variety of cancers.

The combination of multiple immune checkpoint inhibitors as a means for treating cancers has also been emerging quite rapidly. A recent study has shown that the combination of anti–PD-1 mAb (nivolumab) and ipilimumab in patients with previously untreated melanoma resulted in significantly longer progression-free survival than ipilimumab alone (59). Furthermore, the combination of PD-1 and CTLA-4 blockade was able to demonstrate efficacy in patients with PD-L1–negative tumors compared with either agent alone. The expression pattern of B7-H3 contrasts greatly with that of the other checkpoint inhibitors in that the majority of B7-H3 can be found on tumor and tumor-associated tissue, while the others are expressed on immune cells, normal tissue, and cancerous cells. This difference in expression can be highly advantageous for generating not only local responses through the tumor-specific targeting of B7-H3, but also systemic activation of immune cells through additional checkpoint blockade, altogether potentially further enhancing antitumor immunity (Fig. 1F). Despite the fact that no studies are available yet in preclinical models, phase I clinical trials are under way to explore the safety of enoblituzumab in combination with either ipilimumab or anti–PD-1 (pembrolizumab) in patients with refractory cancer (NCT02381314 and NCT02475213).

Radiation is an additional avenue that can be looked at in combination with B7-H3 targeting in a future clinical setting (Fig. 1F). An anecdotal clinical report suggests that ipilimumab plus radiation cooperates to limit melanoma growth (60). Further studies confirmed these results in a small subset of melanoma patients treated with ipilimumab and radiation (61). Of note, resistance was commonly seen and explained by PD-L1 upregulation on the melanoma cells, causing T-cell exhaustion, and highlighting the need for a triple combination therapy. Another area for exploration is the potential synergistic effects of B7-H3 blockade and radiotherapy and its underlying mechanisms for future development of novel cancer immunotherapies (Fig. 1F).

Concluding Remarks

B7-H3 has both immunologic and nonimmunologic functions. Largely overexpressed in human tumor tissues, B7-H3

positively correlates with cancer severity and poor outcome. Compared with other immune checkpoints, the B7-H3 pathway not only regulates innate and adaptive immunity but also promotes cancer cell aggressiveness through various nonimmunologic functions. Therefore, B7-H3 seems to be a unique and interesting target for future cancer immunotherapies. One of the most promising therapeutic strategies may be the use of blocking mAbs against the B7-H3 pathway. Rather than administered alone, blocking mAbs are more likely to achieve synergistic antitumor effects if they are combined with a chemotherapeutic regimen or other checkpoint inhibitors. In parallel, finding its receptor(s) and better elucidating the involvement of the B7-H3 pathway in immune responses and cancer development is crucial, as this knowledge would help with the design of more effective therapeutic agents, with the ultimate goal of complete and durable treatment of human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E. Picarda, K.C. Ohaegbulam, X. Zang
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Biology of Human Tumors

Clinical Cancer Research

B7-H4(B7x)–Mediated Cross-talk between Glioma-Initiating Cells and Macrophages via the IL6/JAK/STAT3 Pathway Lead to Poor Prognosis in Glioma Patients

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Abstract

Purpose: The objective of this study was to evaluate clinical significance and immunosuppressive mechanisms of B7-H4 (B7x/B7S1), a B7 family member, in glioma.

Experimental Design: B7-H4 levels in glioma tissue/cerebral spinal fluid (CSF) were compared between different grades of glioma patients. Survival data were analyzed with Kaplan–Meier to determine the prognostic value of B7-H4. Cytokines from $CD133^+$ cells to stimulate the expression of B7-H4 on human macrophages (Mφs) were investigated by FACS, neutralizing antibodies, and Transwell chemotaxis assay. shRNA, reporter vector, and chromatin immunoprecipitation were used to determine the binding of STAT3 to the B7-H4 promoter. The function of $B7\text{-}H4^+$ Mφs *in vitro* was evaluated through phagocytosis, T-cell proliferation/apoptosis, and cytokine production as well as in the xenografted model for *in vivo* analysis.

Results: We found that B7-H4 expression in tumors was associated with prognosis of human glioblastoma and correlated directly with malignant grades. Mechanistically, glioma initiating $CD133^+$ cells and Mφs/microglia cointeraction activated expression of B7-H4 via IL6 and IL10 in both tumor cells and microenvironment supporting cells. IL6-activated STAT3 bound to the promoter of B7-H4 gene and enhanced B7-H4 expression. Furthermore, $CD133^+$ cells mediated immunosuppression through B7-H4 expression on Mφs/microglia by silencing of B7-H4 expression on these cells, which led to increased microenvironment T-cell function and tumor regression in the xenograft glioma mouse model.

Conclusions: We have identified B7-H4 activation on Mφs/microglia in the microenvironment of gliomas as an important immunosuppressive event blocking effective T-cell immune responses. *Clin Cancer Res; 22(11); 2778–90. ©2016 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Introduction

Glioblastoma (GBM; WHO Grade IV) is the most aggressive and frequent *de novo* intracranial neoplasm in adults, with less than half of patients surviving longer than a year after initial diagnosis. As views changed, more emphasis was placed on the tumor-induced immunosuppression as an important factor of the formation and development of the tumor. Immunosuppressive factors secreted by both tumor cells and microenvironment T-cell infiltrates are proposed to obstruct antitumor immunity (1, 2). Our hypothesis is that the tumor microenvironment cellular interactions between glioma-infiltrating macrophages/microglia (GIM) and glioma cells play a central role in synergistically promoting glioma malignancy and immunosuppression. It has been suggested that tumor-infiltrating macrophages/microglia (TIM) may contribute to the suppression of T-cell–mediated immunity (2, 3). Although some secreted factors (1, 4, 5) and coinhibitory immune molecules (4, 5) have been reported to contribute to the immune regulation in GBM, however, the precise molecular mechanisms underlying these pathways and cellular interaction within the GBM microenvironment are poorly understood.

B7-H4 (also called B7x or B7S1) is a member of the T-cell costimulatory and coinhibitory B7 family (6–8). Functionally, B7-H4 transmits negative signals to T cells to effectively inhibit activation, proliferation, and clonal expansion of $CD4^+$ and

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Translational Relevance

Glioblastoma (GBM) is the most malignant and lethal primary brain tumor. To understand the mechanisms underlying GBM progression is critical for clinical practice. This study revealed the complex immune interactions in the GBM tumor microenvironment involving immune checkpoint B7-H4 and IL6 regulatory mechanisms during tumor progression. Specifically, the results identified that B7-H4$^+$ glioma–infiltrated macrophages (Mφs)/microglia showed immunosuppressive activity, which could be autoregulated by IL6 production. IL6-activated STAT3 bound to the promoter of B7-H4 gene and enhanced B7-H4 expression on Mφs. Our studies demonstrated a direct relationship between the grade of gliomas and the levels of B7-H4 expression, suggesting it as a potential immune-associated marker for progression of gliomas. The findings were further demonstrated using the *in vivo* xenografts model. Overall our study contributes to the understanding of GBM progression, and suggests that targeting the B7-H4 pathway holds therapeutic promise for advanced glioma patients.

CD8$^+$ T cells (6–8). Elevated expression of B7-H4 is detected in human cancer tissues of multiple cancers (9, 10) and is often associated with poor prognosis. We have recently determined the crystal structure of human B7x IgV functional domain and further developed a new cancer immunotherapy with mAbs targeting the B7x IgV (11). Previously, we reported that B7-H4 can be expressed by malignant gliomas (12), but its clinical significance and immunologic role remain elusive. In addition, soluble B7-H4 (sB7-H4) is detected in blood from patients with ovarian, renal cell cancer, hepatocellular carcinoma, osteosarcoma, bladder urothelial carcinoma, and gastric cancer (13–18). However, the relationship between sB7-H4 and malignant grades is still unclear. We have suspected that B7-H4 is related to a subset of tumor-initiating cells in gliomas (12), but details underpinning these observations remain unknown. The evolving understanding of glioma-initiating cells and their importance in tumor pathophysiology (19–25) encourages us to consider that the interplay between glioma-initiating cells and other cell types (e.g., TIMs) may be important for tumor initiation and progression in GBM.

We demonstrated Mφs-modulating cytokine production via the JAK–STAT3 pathway. B7-H4$^+$ GIMs showed immunosuppressive activity and autoregulation by IL6 production. Also, it was observed that adoptive immune therapy of tumor-associated antigen (TAA)–specific T cells in conjunction with TIMs depleted of B7-H4 expression was able to induce tumor regression and prolonged survival of mice in xenograft human gliomas. These results revealed that circumventing the tumor-induced immunosuppression of B7-H4 can induce glioma regression. Overall, our finding indicated B7-H4 as a potential immunity-associated marker of GBM, suggesting that new cancer immunotherapy targeting the B7-H4 pathway holds promise for glioma patients.

Materials and Methods

Preparation of CD133$^+$ glioma cells

To isolate human CD133$^+$ glioma cells, fresh primary GBM surgical specimens were dissociated mechanically and digested with a type IV collagenase (Sigma) for 1 hour at 37°C. A single-cell suspension was then collected on a 30%/70% Percoll gradient and further purified by magnetic separation using anti-CD133 microbeads per the manufacturer's instructions (Miltenyi Biotec), CD133$^-$ cells were also obtained at the same time and cultured at conditions appropriate for growth (continued in Supplementary Methods).

mAb sandwich ELISA detection for sB7-H4, IL6, and IL10

Purified, unlabeled B7-H4–specific mAb (Clone MIH43, 2 μg/mL; AbDserotec) was used for coating and capture using a standard sandwich ELISA method. Thoroughly homogenized tumor tissues were analyzed by IL6 and IL10 ELISA kits (eBioscience). Purified samples were then spotted in duplicate to appropriately coated plates before horseradish peroxidase–conjugated detection antibodies were added. Tetramethylbenzidine was used as a substrate for color development. The optical density was analyzed at 450 nm with a microplate reader (Spectra Max 190; Molecular Devices) and concentrations were quantified using SoftMax Pro software (Molecular Devices).

FlowCytomix detection

Cytokine concentrations in serum, cerebral spinal fluid (CSF) and supernatant of CD133$^+$ and CD133$^-$ cells were measured using FlowCytomix multiple kits according to the manufacturer's directions (eBioscience). Serum was obtained from fresh blood after centrifugation at 3,000 rpm. The supernatant was collected after culture for 72 hours and stored at −20°C. For FlowCytomix detection, the supernatant was added in duplicate to coated 96-well plates. After fluorescent polystyrol beads were coupled with antibodies specific to the cytokines, biotins, and streptavidin-PE were successively added. After being activated (690 nm), samples were analyzed by flow cytometry and quantified with FlowCytomix Pro 2.4 software. Detected cytokines included: MIP-1α, MCP-1, MIP-1β, IL8, RANTES, MIG, IL6, IL10, GM-CSF, and IL4.

Flow cytometry

To detect surface B7-H4, CD11b, or CD133 expression, cells were incubated on ice for 30 minutes with appropriate antibodies or isotype controls (PE-B7-H4, PE-mouse IgG1, APC-CD11b, APC-CD133, APC-mouse IgG1, all from eBioscience, 1:5), washed twice with FACS buffer (PBS containing 0.1% NaN3 and 5% FBS), and resuspended in 0.5 mL 1% formalin/PBS. To analyze intracellular B7-H4, cells were pre-treated with Fix and Perm cell permeabilization reagents (Caltag Laboratories) according to the manufacturer's instructions. Assays of immune function, cell proliferation, cell cycle, apoptosis, cytokines, and cytotoxicity were done under standard methods as previously described. All analyses were performed on a FACS calibur system (Becton Dickinson Immunocytometry Systems).

qRT-PCR

Total RNA from cells was isolated using an RNA purification kit (Qiagen) and treated with RNase-free DNase I to remove genomic DNA (Roche). cDNA libraries were generated using Superscript RNase H-Reverse Transcriptase (Invitrogen) and random hexamers (Sigma-Aldrich). qRT-PCR was performed in an ABI Prism7500 sequence detection system (Applied Biosystems) with SYBR Green Master Mix (Eurogentec) and

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primers (Sangon Biotech Co. Ltd.) at optimized concentrations. Primer sequences for B7-H4 were 5′-TCTGGGCATCCCAAGTTGAC-3′ (forward primer) and 5′-TCCGCCTTTTGATCTCCGATT-3′ (reverse primer), housekeeping GAPDH primers have been described previously. Standard curves were obtained for each gene and the amplification was 90% to 100% efficient. All values were normalized to GAPDH and relative RNA expression was determined and compared to control groups.

Regulation of B7-H4 expression

To regulate B7-H4 expression, Mφs or microglia were cultured for 72 hours with $CD133^+$ cells or $CD133^-$ cells at the ratio of 10:1, $CD133^+$ supernatant or $CD133^-$ supernatant or different concentrations of recombinant IL10, IL6, IL4, and GM-CSF (Peprotech). Neutralizing monoclonal antibodies against human IL6 (anti-IL6, clone 6708, 500 ng/mL) and IL10 (anti-IL10, clone 23738, 50 ng/mL; Peprotech) were used as indicated. For B7-H4 blocking, fresh macrophages were transfected with lentiviral two pairs of short-hairpin (sh)-B7-H4 and sh-mock as directed (Shanghai gene chemical technology limited company, China).RT-PCR and flow cytometry were performed to quantify B7-H4 mRNA and surface protein expression.

In vitro TAA-specific T-cell immunosuppression

Dendritic cells (DC) extracted from peripheral blood mononuclear cells (PBMC) or mouse bone marrow by CD11c microbeads (Miltenyi Biotec) were incubated with irradiated apoptotic U87MG or GL261 cells at a ratio of 1:5 for 24 hours. These tumor-loaded DCs (2×10^4 cells/well) were then used to activate $CD3^+$ T cells from blood or $CD8^+$ T cells from spleen (2×10^5 cells/well) in the presence of 10 U/ml IL2 and 10 ng/ml IL7 for 1 week. To evaluate proliferation, cell-cycle stage, apoptosis, or cytokine secretion, TAA-specific T cells were cocultured with different macrophages at a 1:1 ratio, stained with CFSE, PI, annexin V/PI, or IL2/IFNγ, and sorted by FACS. To measure cytotoxicity, $CD8^+$ T cells isolated from blood was stimulated by macrophages at various ratios and cocultured with CFSE-labeled U87MG cells. FACS was performed with annexin V/PI staining and cytotoxicity was quantified as the percentage of dead or apoptotic cells. For LDH release assay, Mφs/microglias and activated $CD8^+$ T cells (Mφ/microglia:T = 40:1, 20:1, 10:1, 1:1, 1:10, 1:20) were added with or without 1×10^5 GL261 cells per well. After a 12 hours coculture, the supernatant from each well was collected and analyzed with the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega Corporation) and recorded at an absorbance of 490 nm.

Statistical analysis

The two-tailed Student *t* test was performed to assess the significance between experimental groups. The Pearson correlation or linear regression analysis was used for correlations between parameters. Cumulative survival time was calculated by the Kaplan–Meier method and comparison between the groups was tested by the log-rank test. GraphPad software (GraphPad Software, Inc.; version 5.02) was used for all statistical analyses. Statistically significant *P* values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Results

Elevated B7-H4 expression associated with human glioma progression

We examined the relationship between glioma B7-H4 expression and disease progression in the patients of different stages. B7-H4 mRNA levels in glioma tissues with WHO grade 1 to 4 and normal control brain tissue from epilepsy surgery (non-tumor controls) were compared. B7-H4 mRNA expression increased with higher grade tumor stage (Fig. 1A). Furthermore, to verify these findings, the expression of B7-H4 was examined by IHC in 138 glioma and six non-tumor control tissues (tissue microarray, TMA; Supplementary Table S1). We observed immunoreactivity of B7-H4 protein in both the cytoplasm and membrane (Fig. 1B), and a very low level of B7-H4 expression was observed in control compared with glioma tissues. Western blot (WB) analysis showed high expression of B7-H4 protein in high-grade gliomas (HGG), minimal expression in low-grade gliomas (LGG), and negligible detection in non-tumor controls (Fig. 1C). Significant differences for B7-H4 staining pattern were observed between controls and LGG ($P < 0.001$) and between LGG ($n = 33$) and HGG ($n = 105$; $P < 0.001$, Fig. 1D). We searched the Cancer Genome Atlas (TCGA) data GBM gene-expression profile for expression of B7-H4. Compared with normal brain tissue ($n = 10$), B7-H4 overexpressed in GBM tissue ($n = 483$). We futher analyzed the gene expression correlated with GBM phenotype, and found that B7-H4 gene upregulation mainly exists in proneural type (Supplementary Fig. S1).

Flow cytometry was used to assess glioma tissues, $B7\text{-}H4^+CD11b^+$ Mφs/microglia in LGG and HGG were 24.5% and 69.9% (Fig. 1E, $P < 0.01$), respectively. We also assessed B7-H4 in peripheral blood cells ($n = 8$/healthy group, 13/LGG group, 10/HGG group) and glioma specimens ($n = 8$/LGG group, 8/HGG group) was characterized. In blood, $B7\text{-}H4^+CD11b^+$ monocytes in LGG and HGG were 8.5% and 14.4%, (Fig. 1E, $P > 0.05$), respectively.

Prior studies demonstrated a correlative relationship between sB7-H4 and severity of ovarian cancer and renal cancer (13, 14); however, the role of sB7-H4 in human glioma is unclear. We used ELISA to detect sB7-H4 in CSF from 52 patients (clinical characteristics of patients on Supplementary Table S1). We found that increasing concentrations of sB7-H4 in CSF was associated with higher malignancy stages (Fig. 1F). This analysis demonstrated sB7-H4 is released into the CSF of glioma patients and associated with advanced stages in the disease.

Retrospective analysis of B7-H4 expression in GBM to correlate with survival

A large cohort of GBM patients tumors ($n = 70$, Supplementary Table S2) was retrospectively analyzed for B7-H4 expression by IHC to determine the prognostic value. We identified an inverse correlation of tumor cell B7-H4 expression levels (negative vs. moderate vs. strong) and survival in terms of progression-free survival (PFS) and overall survival (OS; Fig. 1G. PFS: $P < 0.001$, OS: $P < 0.001$). This finding suggested a role for B7-H4 as a potential predictor of poor disease outcome.

B7-H4 distribution in both GIMs and tumor cells in glioma tissues

A murine glioma model in C57BL/6 mice was developed to further examine B7-H4 in tumors and GIMs ($CD11b^+$/

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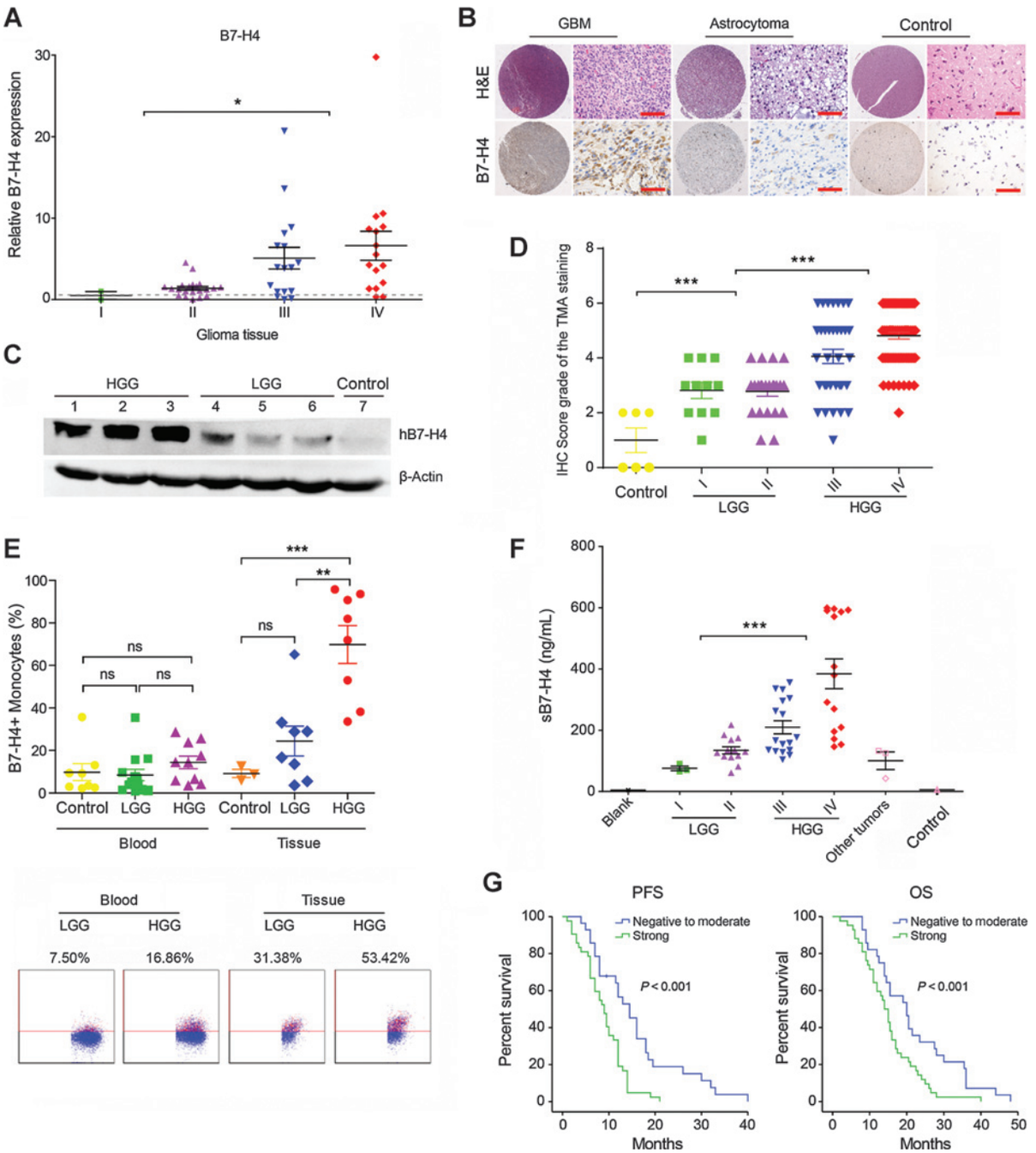

Figure 1.

B7-H4 expression on human gliomas. A, RT-qPCR detection of relative B7-H4 expression in WHO grade 1 to 4 glioma tissues. Differences in mRNA expression in gliomas compared with non-tumor controls (gray line) are shown (n = 2/I, 21/II, 17/III, 16/IV, LGG (I+II) compared with HGG (III+IV), T test, *, $P < 0.05$). B, TMA IHC detection of B7-H4 in clinical GBMs, astrocytoma and control (scale bar, 200 μm). C, WB showing B7-H4 expression in HGG, LGG, and non-tumor controls. Representative results of at least two independent experiments. D, statistics of TMA immunohistochemical detection of B7-H4 in clinical GBMs (n = 6/control, 11/I, 22/II, 35/III, 70/IV, control vs. LGG (I+II): $P < 0.001$, LGG (I+II) vs. HGG (III+IV): $P < 0.001$, t test). E, top, FACS analysis of B7-H4 expression on fresh Mφs/microglia ($CD11b^+$) isolated from peripheral blood or glioma tissue (n = 8/healthy blood for control group, 13/LGG blood group, 10/HGG blood group, 3/non-tumor controls, 8/LGG tissue group, 8/HGG tissue group. The T test, NS, no significance. **, $P < 0.01$; ***, $P < 0.001$). Bottom, data, dot plots of four individuals. F, quantification of ELISA-detected human sB7-H4 concentration in CSF from LGG, HGG and other brain disease (CSF: n = 1/blank, 3/I, 13/II, 17/III, 15/IV, 3/others tumors (2 metastatic tumor and 1 meningioma), 1/control (mild brain trauma). LGG (I+II) vs. HGG (III+IV): T test, ***, $P < 0.001$). Error bars, SEM. G, IHC staining intensity of B7-H4 in tumor niche was evaluated in 70 GBM and used to divide the patients into two groups: negative to moderate expression and strong expression. PFS (left) and OS (right) were then compared between two groups. PFS and OS after the operation were calculated using the Kaplan–Meier method and analyzed with the log-rank test, $P < 0.001$).

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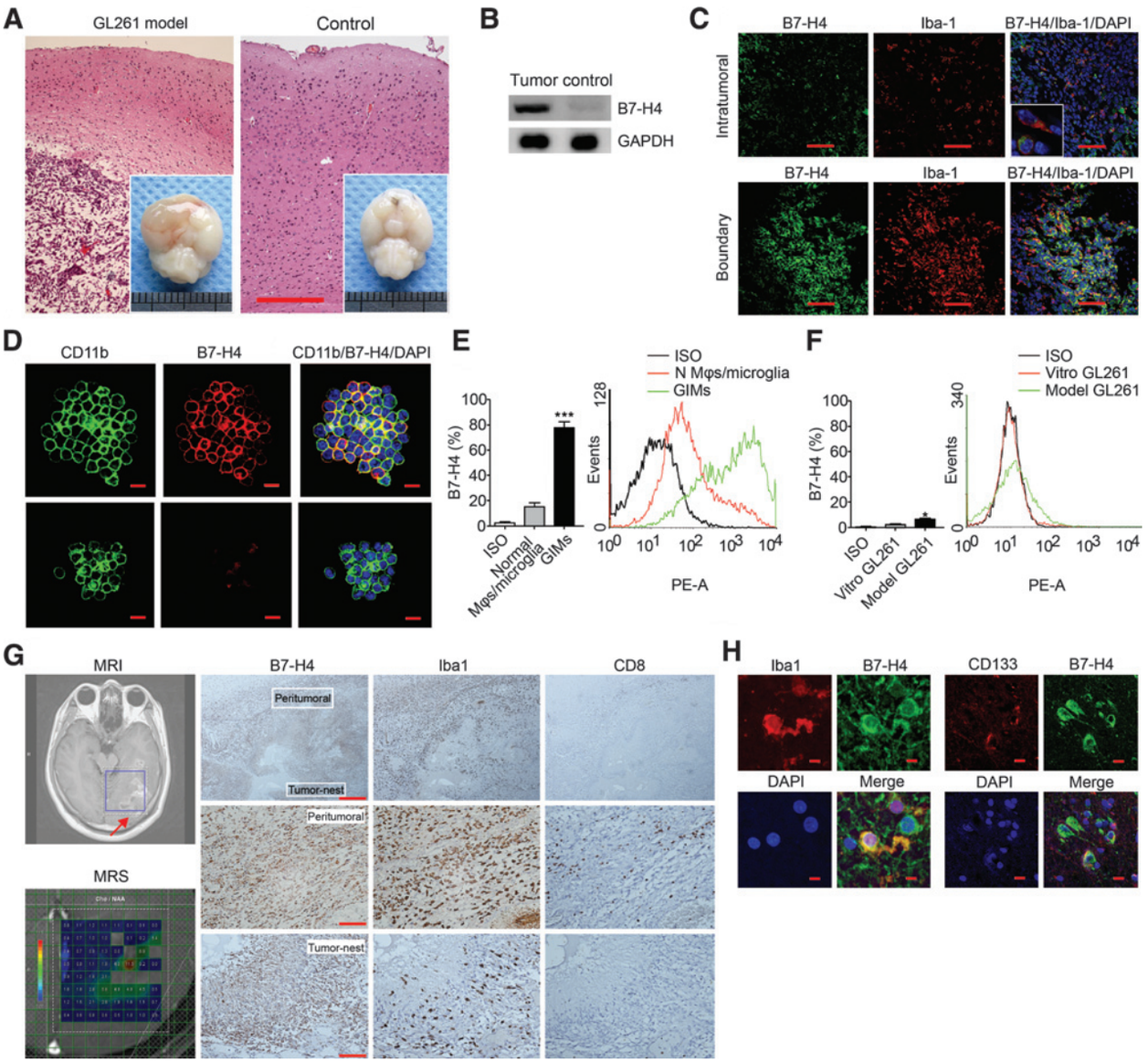

Figure 2.
B7-H4 distribution and localization in glioma, GIMs, and CD133$^+$ cells. A, representative H&E of C57BL/6 mice (20 days after intracerebral injection of 1×10^5 GL261 cells per brain). *Inset*, glioma shown with macroscopic pictures (left). Normal mouse brain images serve as control (right); scale bar, 200 μm. B, WB showing B7-H4 expression in tumor tissues compared with control. C, top, IF staining for B7-H4 and Iba-1 (marker of Mφs/microglia cells) of sections from the intratumoral region. *Inset*, B7-H4$^+$ /Iba1$^+$ GIMs shown at high magnification. Bottom, B7-H4$^+$ GIMs prevalent at the glioma boundary. (88.3% for boundary vs. 38.8% for intratumoral, *T* test, ***, $P < 0.001$; scale bar, 20 μm). D, IF of GIMs from the mouse model (top) and normal microglia from non-tumor control (bottom); scale bars, 10 μm. E, quantification of B7-H4 between normal Mφs/microglia ($n = 10$) and GIMs ($n = 10$) by FACS (*T* test, ***, $P < 0.001$). ISO (isotype, $n = 10$) as control. F, quantification of B7-H4 on *in vitro* cultured GL261 cells ($n = 10$) and *in vivo* model GL261 cells (CD11b$^-$, $n = 10$; *T* test, *, $P < 0.05$). G, representative MRI (top) and MRS (bottom) images of a GBM patient were analyzed. The white and blue boxes indicate the brain regions selected for MRS review. Analysis of B7-H4 expression patterns were correlated with the distribution patterns of GIMs (Iba1$^+$), which localized mostly to peritumoral, or stromal region (red arrow navigated by enhanced-MRI images). Adjacent sections of paraffin-embedded brain tissue from this patient were also stained with B7-H4, Iba1, and CD8. The middle and bottom panels show the stromal and tumor nest at higher magnification (top scale bar, 200 μm, bottom scale bar, 50 μm). H, IF of sectioned human GBM tissues revealed localized B7-H4 expression on GIMs (left) and CD133$^+$ tumor cells (right, scale bar, 20 μm).

CD45$^+$Mφs). Higher levels of B7-H4 expression were found in GL261-derived intracranial tumors (Fig. 2A–B). B7-H4$^+$ GIMs (Iba1$^+$ Mφs) were identified primarily at the glioma tumor boundary (Fig. 2C, 88.3% for boundary vs. 38.8% for intratumoral; ***, $P < 0.001$). We isolated GIM by CD11b magnetic microbeads from the mouse glioma tumor tissues. GIM were further identified in their role in GBM surveillance (26, 27) and found that the isolated cell population of

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GIMs expressed higher levels of surface B7-H4:74% ± 1% for GIMs versus 15% ± 7% for Mφs/microglia from controls (Fig. 2D and E, $P < 0.001$). We compared levels of B7-H4 expression in GL261 cells cultured *in vitro* with GL261 cells implanted into mice and revealed a lower capacity for induction of B7-H4 expression in *in vitro* cultured GL261 cells, 2% ± 1.6% versus 6% ± 2%, $P < 0.05$ (Fig. 2F). To confirm this, GL261 cells from model mouse were isolated and analyzed by WB from *in vitro* cultured GL261 cells as controls. Isolated GIMs from xenografts expressed higher levels of B7-H4 from non-tumor controls (Supplementary Fig. S2).

Clinical tumors were assessed to determine whether the murine tumor observation also show a similar response. MRI and magnetic resonance spectroscopy (MRS) were used to characterize patient intracranial gliomas. For these studies, peritumoral (stromal) and tumor-nest regions were identified (Fig. 2G). Enhanced MRI images-guided brain tissue biopsies taken from these locations were sectioned to include both regions of interest and then employed IHC with anti-B7-H4, cell-specific, anti-Iba1, and anti-CD8 Abs for GIMs (Mφs/microglia), and CTLs, respectively. The IHC analysis revealed higher amounts of B7-H4 on GIMs in the peritumoral regions (Fig. 2G). Immunofluorescence (IF) for GBM tissues proved that not only GIMs but also $CD133^+$ tumor cells expressed B7-H4 as previously shown (Fig. 2H; ref. 12).

Together our results confirmed the presence of both cellular and soluble forms of B7-H4 and established GIMs and glioma cells (including $CD133^+$ cells) as candidates for the sources of this protein. Considering their important role, we further explored the cross-talk between GBM $CD133^+$ cells and GIMs in the glioma microenvironment.

$CD133^+$ cells produce IL6 and IL10 to activate B7-H4 expression on Mφs

We next examined whether GBM $CD133^+$ cells could regulate B7-H4 expression in Mφs. Fresh normal Mφs were incubated in specific culture conditions: control medium only, medium and $CD133^+$ GBM cells, medium and $CD133^-$ GBM cells, and supernatant of $CD133^+$ or $CD133^-$ cells. After 72 hours of incubation, B7-H4 expression on Mφs was assessed by flow cytometry. Mφs cocultured with $CD133^+$ cells expressed higher B7-H4 than those cultured devoid of $CD133^+$ cells (56.1% for $CD133^+$ cell coculture vs. 28.8% for $CD133^-$ cell coculture, $P < 0.05$, Fig. 3A). Mφs exposed to $CD133^+$ cell supernatant also had higher B7-H4 expression than those exposed to $CD133^-$ cell supernatant (49.3% for $CD133^+$ supernatant vs. 16.6% for $CD133^-$ supernatant, $P < 0.01$, Fig. 3A). These findings were supported by RT-qPCR studies of B7-H4 expression of Mφs in $CD133^+$ and $CD133^-$ supernatant (Fig. 3B) in which marked significant elevation of B7-H4 expression was most notable in Mφs cultured in $CD133^+$ supernatant.

We hypothesized that factors secreted by $CD133^+$ cells had a major role in upregulating B7-H4 expression. To test this, FlowCytomix was used to establish cytokine profiles for $CD133^+$ and $CD133^-$ supernatant. Two significant chemokines, MIP-1α and MCP-1, were found to be at substantially higher concentrations in $CD133^+$ supernatant relative to $CD133^-$ supernatant (Fig. 3C, $P < 0.001$). Interestingly, levels of IL6 and IL10 were also elevated in $CD133^+$ media relative to $CD133^-$ supernatant ($P < 0.001$, Fig. 3C). To determine their roles, IL6 and IL10 were added to control cultures of Mφs. We observed a dose-dependent upregulation of B7-H4 expression in Mφs stimulated by these cytokines (Fig. 3D) and verified these findings by suppressing IL6 and IL10 activity with specific Ab, respectively (Fig. 3E).

Because these results suggested a primary role of interleukins IL6 and IL10 in regulating B7-H4 expression that occurs in the microenvironment, we further investigated systemic levels of these cytokines. FlowCytomix was performed with serum and CSF from a series of glioma patients (clinical characteristics in Supplementary Table S1). Only trace amounts of these cytokines were detected in serum and CSF. A relationship between the serologic interleukin levels and tumor grade was not readily delineated; ELISA analysis from tissue homogenates identified elevation of these two cytokines and showed a higher level of IL6 in HGG patients ($P < 0.01$) compared with control patients (Supplementary Fig. S3). This finding supports a role for these two cytokines in GBM and induction of immunosuppression, perhaps more confined to the tumor microenvironment than system-wide. Finally, we used a cell migration assays to examine whether $CD133^+$ GBM cells could initiate recruitment of Mφs/monocytes into the tumor environment. We found enhanced migration of Mφs/monocytes with $CD133^+$ supernatant as a chemoattractant (Fig. 3F, medium-only, $CD133^+$ and $CD133^-$ supernatant: 1 ± 0.00, 5.17 ± 0.78, and 3.12 ± 0.22, respectively).

These findings support a novel mechanism by which $CD133^+$ cells are able to induce Mφs migration into the tumor microenvironment and induce them to express B7-H4 by cytokines IL6 and IL10.

IL6 induces B7-H4 expression via the JAK–STAT3 pathway

Considering that enhanced IL6 was found in glioma tissue homogenates (Supplementary Fig. S3), we then explored the molecular pathways through which B7-H4 expression was regulated. IL6 signaling has been extensively investigated through the JAK–STAT pathway (28), so we hypothesized that the expression of B7-H4 was regulated through this pathway. To confirm this, mouse BV2 microglia cells were stimulated with IL6 and analyzed the phosphorylation of STAT3 by WB. As shown in Fig. 4A, IL6 treatment upregulated phosphorylation of Stat3 and B7-H4 levels in BV2 cells in a dose- and gradient-dependent manner, and 25 ng/mL and 24 hours were suitable dosage and time point. Similar findings were reproduced in the mouse RAW Mφs cell line (Fig. 4B) and Mφs sorted from healthy human peripheral blood (Fig. 4C).

To further determine whether the expression of B7-H4 was regulated by the STAT3 pathway, four pairs of STAT3 shRNAs were individually transduced into BV2 cells to knockdown endogenous STAT3. STAT3 mRNA in BV2 cells transduced with lentiviral stocks expressing shRNA1, shRNA2, shRNA3, or shRNA4 were reduced by 54.2, 65.0, 85.3, and 78.6%, respectively (Fig. 4D, left). Because STAT3 protein was remarkably decreased in the BV2 cells that were transduced with shRNA3, we chose to use shRNA3 for the subsequent experiments. We found that silencing STAT3 with shRNA3 reduced B7-H4 expression with or without the stimulation of IL6 (Fig. 4E). WB analysis of BV2 microglia stimulated with $CD133^+$ supernatant was also performed, which showed similar tendency as IL6 did (Supplementary Fig. S4).

We next determined whether STAT3 could be a transcriptional regulator of the B7-H4 gene. We transfected BV2 microglia cells with a reporter vector encoding Luciferase under

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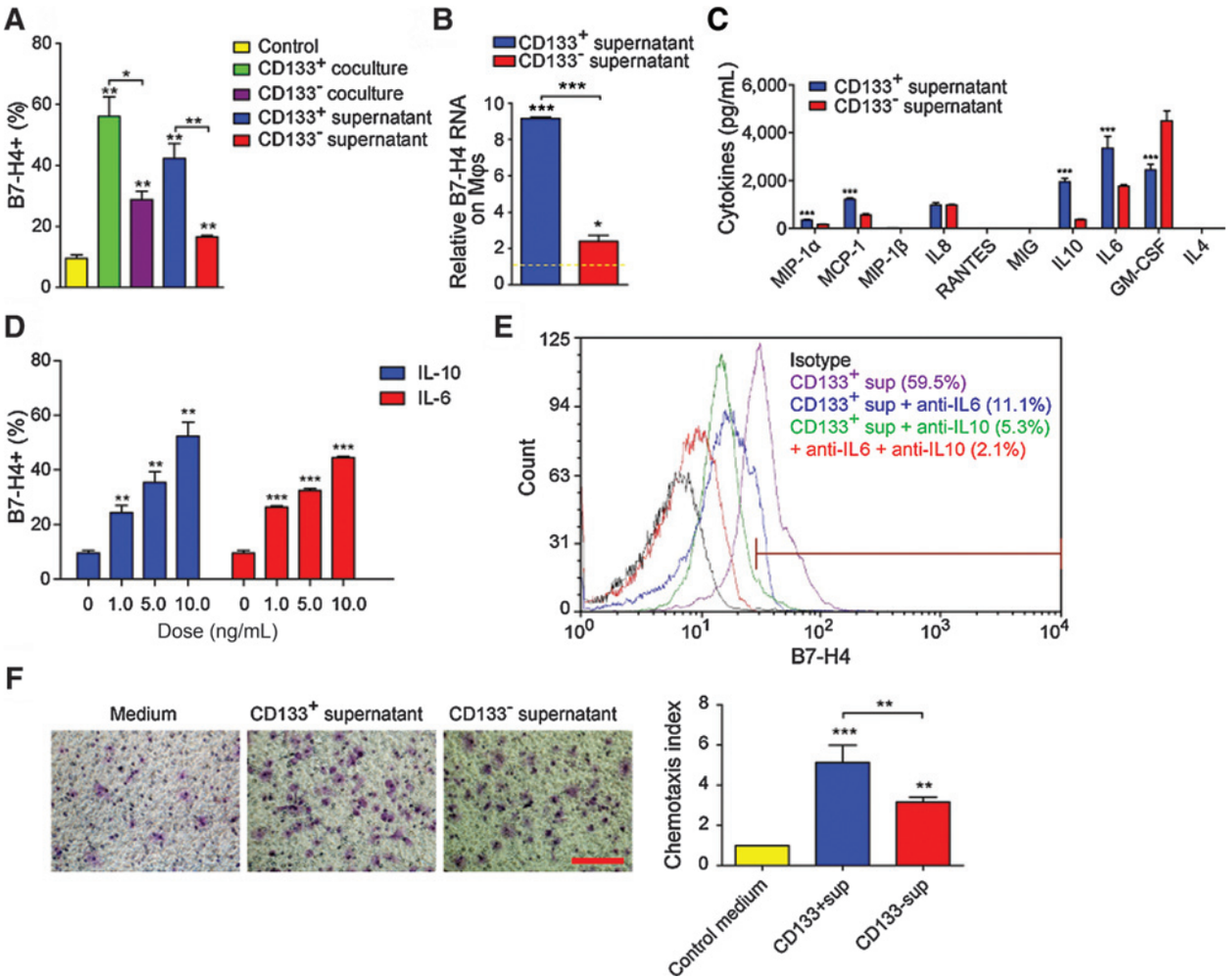

Figure 3.

CD133$^+$ cell-induced normal human Mφs expression of B7-H4 is mediated by IL6 and IL10. A, freshly isolated healthy human Mφs were incubated with control medium, CD133$^+$ cells, CD133$^-$ cells isolated from human GBM tissue or supernatant from them for 72 hours and then analyzed them for B7-H4 expression by FACS (*T* test, *, $P < 0.05$; **, $P < 0.01$.). B, RT-qPCR for relative B7-H4 gene expression on Mφs cultured in supernatant from either CD133$^+$ or CD133$^-$ cells compared with control (yellow line; *T* test, *, $P < 0.05$; ***, $P < 0.001$). C, quantity of various cytokines in the supernatant of CD133$^+$ cells or CD133$^-$ cells examined by FlowCytomix (*T* test, ***, $P < 0.001$). D, B7-H4 expression on Mφs was detected by FACS analysis after they were treated with 0, 1.0, 5.0, or 10.0 ng/mL recombinant IL10 or IL6 for 72 hours (*T* test, **, $P < 0.01$; ***, $P < 0.001$). E, fresh normal Mφs were cultured with supernatant from CD133$^+$ cells for 72 hours in the presence or absence of neutralizing antibodies against IL6 (500 ng/mL) and/or IL10 (50 ng/mL) followed by FACS analysis. F, left, cell invasion capabilities of normal human peripheral Mφs following culture in either supernatant from CD133$^+$ or CD133$^-$ cells, or control medium were analyzed using 6.5 mm Transwell with 8.0-μm pore polycarbonate membrane insert 24 hours later; scale bar, 100 μm. Right, quantitative results for chemotaxis index are showed. Data (±SEM), three independent experiments with similar results. Paired *t* test, $P < 0.01$, ***, $P < 0.001$.

control of the B7-H4 promoter (PGL3-B7H4.WT). Phosphorylation of STAT3 upregulated B7-H4 promoter activity, which was abrogated by knockdown of STAT3 with shRNA (Fig. 4F). These studies suggested that STAT3 regulated B7-H4 transcription via enhancing the B7-H4 promoter. We then carried out chromatin immunoprecipitation (ChIP) assays to assess whether STAT3 directly binds to the B7-H4 promoter. As shown in Fig. 4G, STAT3 protein bound to the B7-H4 promoter was significantly increased in BV2 microglia cells treated with IL6. The epistatic relationship between the expression of B7-H4 and IL6–STAT3 pathway (Supplementary Fig. S5) may provide a new line of evidence for the development of STAT3 inhibitor as anticancer chemotherapy drugs.

CD133$^+$ cells mediate immunosuppression through B7-H4$^+$ Mφs *in vitro* and *in vivo*

To explore the role of B7-H4 in CD133$^+$ cell-mediated immunosuppression, CD133$^+$ cells were sorted from human GBM cell suspensions, and the supernatant of CD133$^+$ cell culture was then used to treat normal Mφs to induce expression of B7-H4. We found that CD133$^+$ cell supernatant-treated Mφs showed markedly reduced uptake of red fluorescent tag–labeled polystyrene latex beads compared with CD133$^-$ cell supernatant-treated Mφs (Fig. 5A), suggesting that the supernatant of CD133$^+$ cells inhibits the phagocytotic activity of Mφs. We then examined the function of B7-H4 in Mφs induced by the supernatant of CD133$^+$ cells. B7-H4 expression in Mφs

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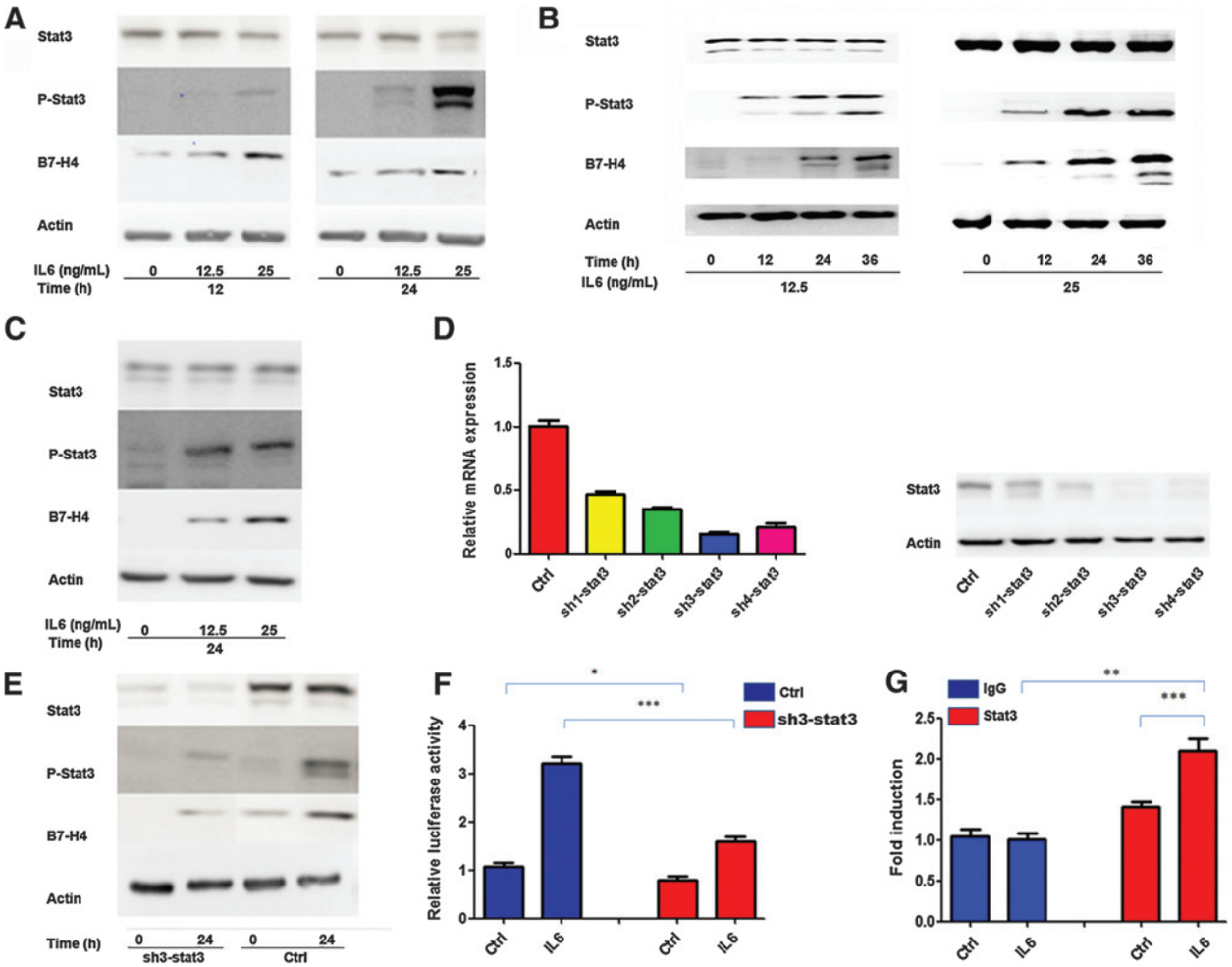

Figure 4.

STAT3-dependent B7-H4 expression in microglia/Mφs cells. A, WB analysis of B7-H4 and Stat3 phosphorylation in the mouse BV2 microglia cell line with different time intervals and dose gradient of IL6. B, similar findings were reproduced in the mouse RAW Mφs cell line by WB. C, WB analysis of B7-H4 and Stat3 phosphorylation in healthy human peripheral macrophages after incubation for 24 hours with different doses of IL6. D,Stat3 mRNA expression in the BV2 microglia cells infected with control, shRNA1, shRNA2, shRNA3, and shRNA4. All shRNAs remarkably downregulated Stat3 mRNA (left) and protein levels (right). ShRNA3 was the strongest one, and the other shRNAs had less effect. E, the protein expressions of phosphorylated Stat3 and B7-H4 in BV2 microglia cells infected with shRNA3 or control after being stimulated with IL6 (25 ng/mL). ShRNA3 significantly inhibited phosphorylated Stat3 and B7-H4 protein expression after being stimulated with IL6. F, BV2 microglia cells were transfected with the above shRNA3 plasmids or control shRNA for 24 hours, and the luciferase activity was measured (*T* test, *, $P < 0.05$; ***, $P < 0.001$). G, two Ab (anti-IgG and -STAT3) were used in the ChIP assays using the BV2 microglia cell line treated with or without IL6 (*T* test, **, $P < 0.01$; ***, $P < 0.001$).

was silenced by shRNA (Fig. 5B) and Mφs were cocultured with T cells activated by U87MG-loaded CD11c$^+$ DCs. We found that the B7-H4 silencing in Mφs increased T-cell proliferation (Fig. 5C) and decreased T-cell apoptosis (Fig. 5D). Correspondingly, the B7-H4 silencing also reduced T cells that did not produce IL2 or IFNγ (Fig. 5E). Furthermore, B7-H4$^+$ Mφs cultured in CD133$^+$ supernatant inhibited CTL-cytotoxic activity against U87MG glioma cells. However, the inhibitory effects were mitigated after B7-H4 expression was blocked (Fig. 5F).

To investigate the function of B7-H4 in Mφs *in vivo*, we used the GL261-induced glioma mouse model. GL261 tumor cells derived from mouse glioma contained CD133$^+$ cells and were able to inhibit both CD8$^+$ and CD4$^+$ T-cell function *in vitro* (Supplementary Fig. S6). Interestingly, the suppressive capacities were partially reversed by exposing Mφs to anti–B7-H4 Ab (Supplementary Fig. S6). These results suggested that CD133$^+$ cells can induce B7-H4 expression on Mφs, which leads to T-cell inhibition.

Suppression of B7-H4 leads to T-cell activation and tumor regression in the xenograft glioma model

NOD/SCID xenograft gliomas derived from U87MG cells were further investigated. TAA-specific CD8$^+$ T cells (CTL) were injected i.v. together with: (i) normal Mφs (M), (ii) CD133$^+$ glioma-initiating cells conditioned Mφs (GCM), (iii) conditioned Mφs transduced with sh1-mock, or (iv) conditioned Mφs transduced with sh1-B7-H4 to silence B7-H4 expression. We observed tumor growth inhibition in mice injected together with CTL+M and CTL+ GCM+ sh1-B7-H4 Mφs, whereas the other two groups showed rapid progression of nearly 200 mm^3 per day (Fig. 5G

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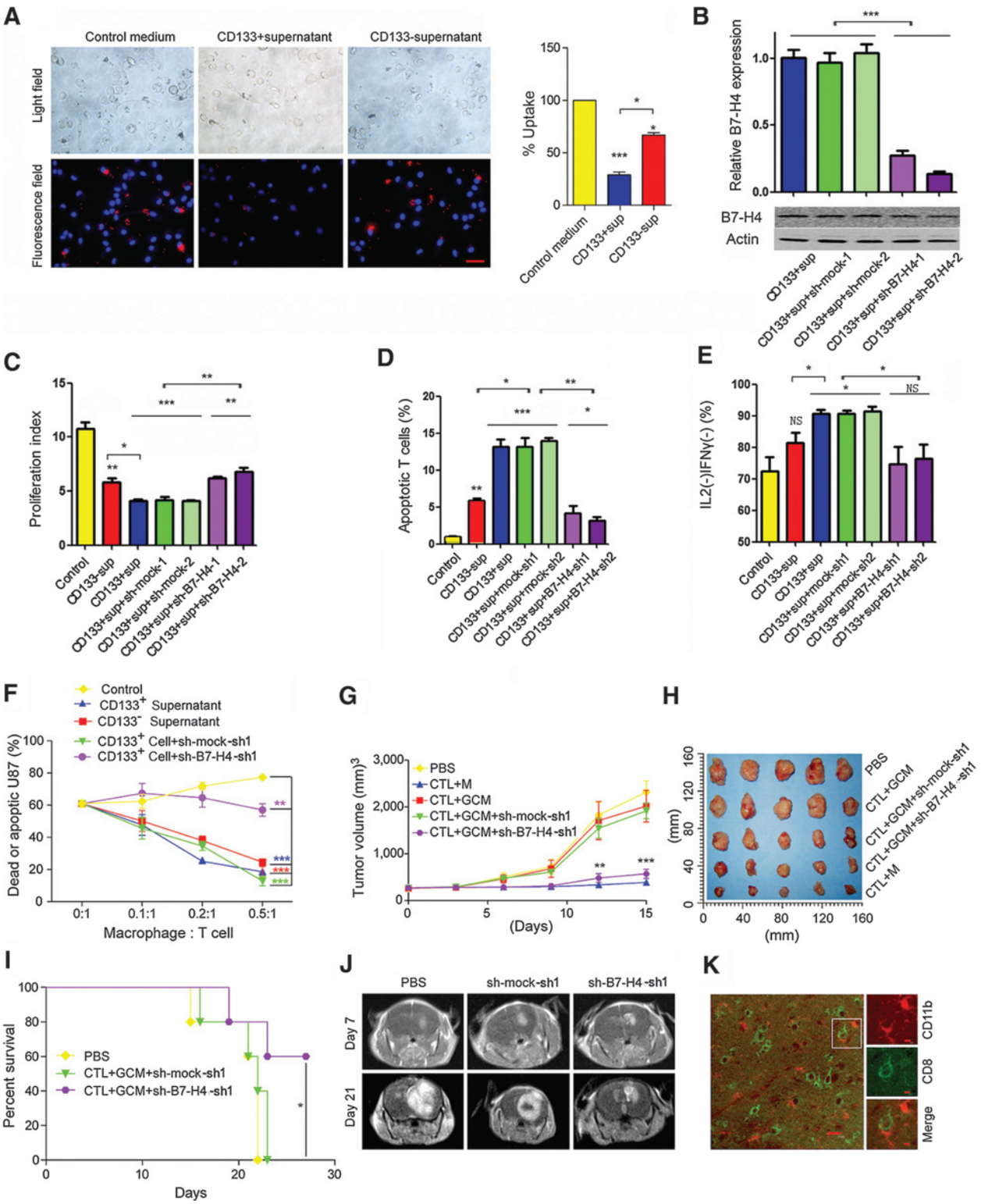

Figure 5.

CD133$^+$ cells mediate immunosuppression through B7-H4$^+$ Mφs *in vitro* and *in vivo*. A, left, normal Mφs were expanded in either standard medium, supernatant from CD133$^+$ cells, or supernatant from CD133$^-$ cells with latex beads (red) before DAPI (blue) staining and phagocytosis assay. Using light and fluorescence microscopy (right, scale bar, 50 μm), the percentage of cell-mediated uptake (phagocytically active cells/total cells) was determined (paired *t* test, *, $P < 0.05$; ***, $P < 0.001$). B, qRT-PCR and WB showed blocking effects of two pairs of sh-B7-H4 and sh-mock lentivirus in CD133$^+$ conditioned Mφs (*T* test, *, $P < 0.05$; ***, $P < 0.001$). (*Continued on the following page.*)

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and H). These results suggested that CD133$^+$ cells played a primary role in driving B7-H4$^+$ Mφs to reduce TAA-specific T-cell immunity *in vivo*.

To determine whether B7-H4 could serve as a predictor of prognosis, we infused CTLs with CD133$^+$ glioma-initiating cells conditioned sh1-mock or sh-B7-H4 Mφs via a caudal vein injection of mice with U87MG-derived intracranial tumor. Mice with sh1-B7-H4–treated Mφs survived significantly longer than the sh1-mock group (Fig. 5I). In contrast, mice infused with PBS and sh1-mock–treated Mφs had U87MG cell invasion, which was also characterized by early lesions detected by MRI (Fig. 5J). Supporting the role of Mφs in regulating T-cell function, human CD11b$^+$ Mφs/microglia and human CD8$^+$ T cells appeared to comigrate into intracranial human tumors (Fig. 5K).

GIMs induce B7-H4 expression in CD133$^+$ cells and autoregulation through IL6

GIMs were isolated from murine GL261 tumors to investigate their effects on CD133$^+$ cells. Using a Transwell chemotaxis assay in a coculture system, migration abilities between GIMs and normal Mφs/microglia (NM) were compared. GIMs exhibited higher time-dependent migration abilities (Fig. 6A).

Next, GL261-derived enriched CD133$^+$ cells were separated from CD133$^-$ cells, and both cell populations were cultured in GIM-conditioned media. GIMs upregulated B7-H4 expression in CD133$^+$ cells and CD133$^-$ cells according to flow cytometry, IF, and WB analysis (Fig. 6B–D). Considering IL6 and IL10 were involved in B7-H4 induction, we measured the level of these cytokines in the sera of: (i) normal mice, (ii) CD133$^-$ cells initiated tumor mice, (iii) CD133$^+$ cells initiated the tumor group, and supernatants groups from NM and GIM cultures. There was no observable difference in serum IL6 levels across the three groups. However, we observed that GIM-secreted IL6 exceeded NM-secreted IL6 by >15-folds (Fig. 6E). No difference was observed for IL10 concentrations in these groups (data not shown). These results support a role for IL6-promoting B7-H4 expression in the tumor microenvironement. Finally, we also find that IL6 can upregulate B7-H4 expression in mice NMs *in vitro* as human Mφs did (data not shown). The results showed that Mφs/microglia in the tumor environment had markedly enhanced B7-H4 expression in the presence of CD133$^+$ glioma-initiating cells.

Discussion

Immunosuppressive processes occurring both systemically and locally in the tumor microenvironment have limited the normal function of the immune system in primary brain malignancies. Our studies characterizing the immunologic roles of GIMs, tumor-derived cytokines, and tissue-derived glioma-initiating cells have revealed evidence for an immunosuppressive role of B7-H4 in human malignant glioma.

We found that increasing B7-H4 expression associated with progression of human glioma. Notably, the distribution of B7-H4–expressing cells was much higher at the peritumoral edges (i.e., stroma) when compared with the tumor nest. What was important, our studies demonstrated that PFS and OS are negatively correlated with expression of B7-H4 in human gliomas. Expression of B7-H4 was detected on the surface of CD11b$^+$/Iba$^+$ monocytes, microglia, and macrophages. CD8$^+$ T cells have been shown to orchestrate autoimmunity in immune privileged locations (29) and antitumor immunity in malignancy (30, 31). Interestingly, we found that CD8$^+$ T cells were also mainly present at the peritumoral edges. Colocalization of B7-H4, Mφs/microglia, and CD8$^+$ T cells at peritumoral edges suggests a functional connection in the tumor microenvironment on the periphery, but not internally. We found significantly higher levels of IL6 in the HGG group compared with LGG or LGG group compared with the normal, but not in serum, suggesting that the immunosuppressive effects of the cytokines are limited to the tumor environment.

B7-H4 in the tumor microenvironment, surrounding stroma and invasive edge, may contribute to the escape from immune surveillance during tumor cell invasion into adjacent brain tissue. We believe that recruitment of monocytes/Mφs to the tumor at the glioma site occurs in the malignant state (32, 33). The development of these TIMs is stimulated by "polarization" induced by the glioma and other tumor cells, resulting in TIMs with a tumor-promoting phenotype (M2). A chemokine-mediated process that spawns tumor development in the microenvironment is demonstrated (27, 34, 35). It is also demonstrated that microglia derived from non-glioma human subjects could curb glioma cell growth by the secretion of chemokine like IL8 and MCP-1, but microglia and monocytes cultured from glioma patients were inefficient at reducing the sphere-forming capacity of tumor cells (36). Our studies showed that glioma-initiating cells mediated expression of B7-H4 on Mφs. Moreover, the detection of Mφ-recruiting chemokines, MIP-1α and MCP-1, which are secreted by CD133$^+$ glioma-initiating cells, presents evidence to support a process whereby Mφs are signaled to invade the tumor microenvironment.

Our experimental findings demonstrated that normal monocytes/Mφs were induced to express B7-H4 via IL6 and IL10 by human CD133$^+$ cells (Fig. 3) or U251 glioma-initiating cells (our published data; ref. 37). In turn, a suppressive potential conferred by Mφs-expressing B7-H4 contributed to immunopathology

(*Continued.*) C to F, tumor-specific CTLs, activated by U87MG-loaded CD11c$^+$ DCs, were cocultured separately with pre-treatment (described above) Mφ groups, and Mφ groups in supernatant from CD133$^+$ cells transfected with two pairs of sh-B7-H4 or sh-mock lentivirus. Immunoreactivity, specifically CTL proliferation (**C**, CFSE), apoptosis (D, Annexin V/PI staining), and finally IL2 and IFNγ expression (E) were measured by FACS (*T* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). F, tumor-specific CTLs (CD3$^+$) conditioned with different ratios of pretreated Mφs (described above) were sorted and cocultured with CFSE-labeled U87MG cells (10:1). U87MG cells were stained with Annexin V/PI to show the percentage of apoptosis or death (72 hours; *T* test, **, $P < 0.05$; ***, $P < 0.01$). G, human U87MG tumors in SCID/NOD mice were measured after injection of 6×10^6 TAA-specific CTLs and different Mφ preparations (day 12) and excised 15 days after injection ($n = 5$/group; *T* test, **, $P < 0.05$; ***, $P < 0.01$). H, tumor size was measured 15 days after injection ($n = 5$/group). I, intracranial tumor mouse survival was determined following growth/treatment of lesions arising in the right striatum. Treatment entailed intracaudal vein injection (day 7) of 6×10^6 TAA-specific CTLs and 3×10^6 CD133$^+$ cells conditioned Mφs transfected with (i) sh1-mock (CTL+GCM+sh-mock), (ii) sh1-B7-H4 (CTL+GCM+sh-B7-H4), or (iii) PBS ($n = 5$/group). All lesions were characterized by MRI at 7 and 21 days (Kaplan–Meier survival analysis, *, $P < 0.05$; J) and stained by IF. IF-stained sections show human CD11b$^+$ Mφs/microglia and human CD8$^+$ T cells colocalizing in three independent experiments (K); scale bars, (left) 20 μm, (right) 5 μm.

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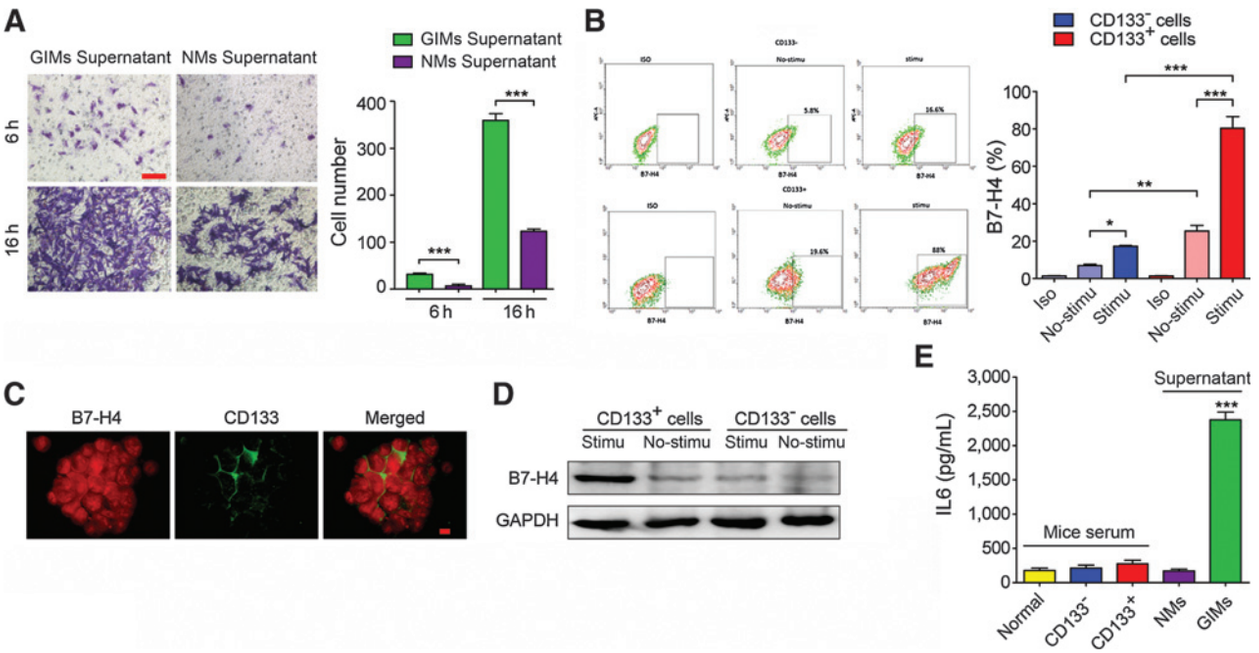

Figure 6.
GIMs stimulate CD133$^+$ cell B7-H4 expression and autoregulation through IL6. A, left, serial time points of Transwell chemotaxis assay were used to demonstrate the recruitment of GL261 tumor cells in the supernatant of GIMs and normal Mφs/microglia (NMs); scale bar, 10μm. Right, quantification of immigrated GL261 cells. Data, means ± SE of three independent experiments ($n = 3$, t test, ***, $P < 0.001$). B, left, FC showing B7-H4 expression on CD133$^-$ cells and CD133$^+$ cells cultured *in vitro* with supernatant from GIMs. Right, quantification of B7-H4 level on CD133$^-$ cells and enriched CD133$^+$ cells upon supernatant stimulation (GIMs from 6 enriched CD133$^+$ cells initiated glioma models/group, T test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). ISO (isotype) as control. C, IF staining for stimulated enriched CD133$^+$ cells. Representative images of three independent staining experiments are shown; scale bar, 20 μm. D, WB analysis of B7-H4 protein in enriched CD133$^+$ cells and CD133$^-$ cells upon GIMs supernatant. E, quantification of ELISA-detected IL6 levels in the serum of normal mice, and mice with CD133$^-$ cells or enriched CD133$^+$ cells initiated glioma and supernatant from normal Mφs/microglia as well as GIMs ($n = 10$, t test; ***, $P < 0.001$).

based on several lines of evidence. First, forcing B7-H4 expression on glioma-initiating cells impaired phagocytosis in normal Mφs, inhibited T-cell proliferation and cytokine production, and led to reduced cytotoxicity of T cells. Second, blocking B7-H4 expression significantly impaired the Mφs' suppressive capacity. Third, despite the presence of potent TAA-specific effector T cells *in vivo*, CD133$^+$ cell-conditioned Mφs promoted intracranial and subcutaneous tumor growth in SCID/NOD mice bearing human gliomas. Colocalization of human CTL and Mφs/microglia *in vivo* in our mouse model presents new evidence for the role of B7-H4$^+$ GIMs in immunomodulatory function.

Antitumor effects appear to be obstructed by a dichotomous mechanism that eliminates M1 macrophage-mediated innate immune responses and impairs T-cell activation. We observed that CD133$^+$ cells stimulated by GIMs, which secreted high levels of IL6, expressed B7-H4 but did not secrete notable amounts of IL6 or IL10 into sera. Functionally, IL6 resulted in B7-H4 expression in Mφs. Other studies also demonstrated that IFNγ could upregulate B7-H4 expression on mouse embryo fibroblasts, but detailed pathway was unknown (38). It seems plausible that glioma-initiating cells native to the tumor microenvironment trigger immunosuppression in part by "recruiting and talking" to Mφs. Following an initial exchange with the GSGs, the Mφs became polarized to the M2 type and integrated into an external role in the developmental stage of this immunomodulatory process and conducted persistent "B7-H4 education" through autoregulation. This intimate process is further promoted by Glioma initiating cells and fosters the immunosuppressive conditions mounted in the tumor stroma. The immunosuppressive association between glioma-initiating cells and GIMs may thus be defined in the context of time (early vs. late) and space (niche-inhabiting vs. recruitment and marginalization) to explain the proposed role of B7-H4 as a potential immunity-associated marker for malignancy progression and prognosis (Supplementary Fig. S7).

In this study, we showed that CSF sB7-H4 concentration tended to correlate with malignancy grades of glioma. The blood–brain barrier (BBB) normally allows the permeability of small molecules (400–500 Da) and some small lipid-soluble proteins (39), B7-H4 (50–80 kDa) may be too large to pass across this natural barrier. Considering CSF comes in direct contact with the extracellular compartment of the central nervous system and the existence of BBB, CSF sB7-H4 may be a better potential immunity-associated marker for supratentorial tumors like gliomas when compared with serum sB7-H4. Enrolling more research subjects with intracranial immunologic diseases to reflect the enhanced utility of sB7-H4 in CSF over sB7-H4 from serum may be needed.

In summary, our studies demonstrated a direct relationship between WHO grading of gliomas and the levels of B7-H4, suggesting its utility as a potential immunity-associated marker for progression of the disease. We established crucial roles of

B7-H4 in the cellular interplay of glioma-mediated immunosuppression between $CD133^+$ glioma-initiating cells and TIMs/GIMs, and uncovered that such a "cross-talking" contributed to glioma pathology. This study revealed a previously unrecognized immune evasion route and provided an anti–B7-H4 therapeutic target in patients with malignant gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Yao, C.C. Chen, K. Jin, X. Zang, Y. Mao
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yao, H. Ye, L. Mo, Q. Yue, A. Baral, L. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Ye, L. Mo, Q. Yue, C.C. Chen, W. Hua, J. Zhang, K. Jin, Y. Mao
Writing, review, and/or revision of the manuscript: Y. Yao, Z. Qi, D.S.B. Hoon, J.C. Vera, J.D. Heiss, C.C. Chen, X. Zang, Y. Mao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yao, Z. Qi, Y. Wang, L. Zhou
Study supervision: Y. Mao

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B7-H4 (B7x)-mediated cross-talk between glioma initiating cells and macrophages via the IL-6/JAK/STAT3 pathway lead to poor prognosis in glioma patients

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Supplemental Data

Supplemental Experimental Procedures

Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6

Supplementary Table 1
Supplementary Table 2

Supplemental Experimental Procedures

Specimens and TMA, patients and clinical follow-up. Fresh tumor specimens, cerebrospinal fluid and peripheral blood were obtained from patients undergoing craniotomy at the Department of Neurosurgery, Huashan Hospital (Shanghai, China). Normal PBMCs were obtained from healthy blood donors attending the Shanghai Blood Center, all of whom were negative for antibodies against hepatitis B virus, hepatitis C virus, HIV, and syphilis. The Huashan Hospital IRB approved the protocol for this study (KY2011-190).

For TMA, 138 samples were acquired from the patients who underwent radical resection between 2002 and 2009. These included tissue sections from 70 patients with WHO Grade IV astrocytoma (GBM), 35 patients with Grade III (anaplastic) astrocytoma,22 patients with Grade II (diffuse) astrocytoma, 11 patients with Grade I (pilocytic) astrocytoma (Supplementary Table 1). These also included 6 sections of brain tissue from trauma surgery and etc. for control. Histopathological diagnosis was performed by experienced neuropathologists according to World Health Organization (WHO) guidelines (1). Of them, all the GBM patients received radiotherapy (fractionated focal irradiation 2 Gy/day, 5 days/wk for 6 wks, 60 Gy totally) and temozolomide therapy (75 mg/m2 day, 7 days/wk throughout radiotherapy, followed by 150–200 mg/m2 for 5 days during each cycle, 28 days per cycle for 6 cycles) postoperatively. Informed consent was obtained from all patients. OS and PFS were determined on September 30, 2012. OS was taken as the date from primary surgical treatment to death or the last follow-up date. PFS was defined as the time interval from diagnosis to the first tumor progression or recurrence defined by MRI scans.

IHC analysis of TMA. Paraffin embedded sections of brain tissue were prepared for IHC as previously described and subsequently stained with primary mouse monoclonal antibody(Ab) to B7-H4 (AbD Serotec, 1:50), rabbit polyclonal Ab to Iba1 (Wako; 1:250) o rabbit polyclonal Ab to CD8 (Epitomics, 1:500) overnight at 4°C. For TMA, samples were fixed in 10% formalin solution (Sigma-Aldrich) for 24 hr at 4°C within 24 hr after surgery and then paraffin-embedded. A representative area of each GBM was marked on an H&E section of each patient's paraffin block avoiding necrosis and extensively vascularized area. A corresponding tissue core of 2 mm diameter was extracted from the

original donor block using an arraying machine (MTA-1, Beecher Instruments). The cores were fit into a vertical hole that was bored in a recipient paraffin block. Recipient blocks were incubated at 58°C for 5 min, pressed on a hot plate for 3 min, and cooled in ice water to enable tissue cores to integrate into the recipient block. Sections of 4 μm thickness were cut from each array block. Immunohistochemical analysis of 4-um TMA sections was carried out as previously described (2). In brief, the TMA slides were dewaxed, rehydrated, antigen retrieval, blocked and then incubated with B7-H4 antibody (1:100 dilution; Cell Signaling Technology, Beverly, MA) at 4°C overnight. The sections were then incubated with biotin-labeled secondary Ab and streptavidin-peroxidase for 30 min each (DAKO Diagnostics). The samples were developed with 3, 3-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) and counterstained with hematoxylin. Then, the slides were dehydrated following a standard procedure, and sealed with coverslips. For evaluation, as mentioned previously (2), the B7-H4 staining in TMAs was examined by two independent blinded observers simultaneously, and a consensus score was reached for each core. The positive reaction of B7-H4 was scored into four grades according to the intensity of the staining: 0, 1, 2, and 3. The percentages of B7-H4–positive cells were also scored into four categories: 0 (0%), 1 (1% to 33%), 2 (34% to 66%), and 3 (67% to 100%). In the cases with a discrepancy between duplicated cores, the higher score from the two tissue cores was taken as the final score. The sum of the intensity and percentage scores is used as the final staining score. The staining pattern of the biopsies was defined as follows: 0, negative; 1 to 2, weak; 3 to 4, moderate; 5 to 6, strong.

WB analysis. Brain tissues were thoroughly homogenized into protein lysate as previously described[3]. After separation by SDS-PAGE, proteins were electrotransferred onto nitrocellulose membranes and incubated successively with blocking solution, rabbit monoclonal Ab to B7-H4 (1:5000; Epitomics, California), and HRP-linked secondary Abs. Images were documented with Image-Pro Plus software (version 5.1; Media Cybernetics, Silver Spring, MD, USA).

Immunofluorescence analysis. Paraffin-embedded or frozen brain tissue was stained with either rabbit polyclonal Ab to CD133 (Abcam; 1:250), rabbit polyclonal Ab to Iba1 (Wako; 1:250), mouse Ab to B7-H4 (AbD Serotec, 1:50), rabbit Ab to B7-H4

(CUSABIO; 1:50), mouse Ab to CD11b (Abbiotec; 1:200) or rabbit polyclonal Ab to CD8 (Epitomics; 1:500). Primary Abs were then subjected to the following fluorochromes for 30min: Alexa Fluor (AF) 594-conjugated donkey anti-rabbit IgG, AF 594-conjugated donkey anti-goat IgG, AF 594-conjugated donkey anti-mouse IgG, AF 488-conjugated donkey anti-mouse IgG1 and AF 488-conjugated donkey anti-rabbit IgG1 (Invitogen; 1:500).

Cell lines and preparation of CD133+ glioma cells. CD133- cells were cultured in DMEM medium with 10% FBS and the CD133+ cells were cultured in vitro with a neurosphere medium consisting of DMEM/F12, B27 (Gibco), EGF (20 $ngmL^{-1}$; Chemicon, San Diego, CA, USA) and FGF-2 (20 $ngmL^{-1}$; Chemicon). U87MG and GL261 cells ,RAW cells, BV2 cells （obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences）were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37□ with 5% CO_2. From GL261 cell line, CD133+ cells were enriched from purified and dense colonies and cultured them according to human glioma cell culture conditions described above.

Isolation of Mφs /microglia from peripheral blood and tissues. PBMCs were obtained by centrifugation on a Ficoll-Hypaque density gradient (Sigma-Aldrich). For inducing Mφs, monocytes from healthy donors were purified using CD11b microbeads per manufacturer's directions (Miltenyi Biotec) and cultured for 5 days in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) with 50 UmL^{-1} granulocyte-macrophage colony-stimulating factor (GM-CSF; eBiosciences) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For microglia, fresh human or mouse brain tumor tissues (3g tissue/tube) were successively minced with sterile scalpels, crumbed in a glass homogenizer (glass Potter, Braun, Melsungen, Germany) and filtered through a pre-moistened 40 μm cell strainer (BD Biosciences 352350) and collected into a 50mL centrifugal tube. After mechanical dissociation, the cell suspension was purified using a Percoll gradient (consisting of Percoll 70, 30%, and HBSS) and centrifuged for 30 min at 500g. The interface was collected, rinsed, and purified by CD11b microbeads.

Stat3 targeting short hairpin RNA, The short hairpin RNA (shRNA) sequences were designed using Invitrogen online BLOCK-iT RNAi Designer

(http://www.invitrogen.com/rnai). The targeting sequences of shRNAs on STAT3 used in the experiments were 5'-GCAACAGATTGCCTGCATTGG-3' (shRNA1), 5'-GCCTCTCTGCAGAATTCAAAC- 3' (shRNA2), 5'-GGTACATCATGGGCTTTATCA -3' (shRNA3), and 5'-GCACCTTCCTGCTAAGATTCA -3' (shRNA4). The Stat3 targeting shRNA sequences or mock sequences were inserted into the PLKO vector. Lentiviral stocks were generated by transfecting the PLKO constructs expressing shRNA targeting STAT3 into mouse BV2 microglia cells, followed by harvesting after 48 h. Efficiency of lentiviral-mediated shRNA knockdown of STAT3 in cells was determined by Western blot analysis and real-time PCR.

Transient transfections and luciferase assays. The human B7-H4 promoter was cloned into pGL3 plasmid (Promega), named pGL3-B7-H4-promoter. BV2 cells were transfected using lipofectamine 2000 reagent with 100 ng of pGL3-B7-H4-promoter according to the manufacturer's instructions. IL-6 was used to stimulate the transfected cells after 24hr. Reporter assays were performed 48h post-transfection using the Dual-luciferase assay system (Promega), normalized for transfection efficiency by co-transfected Renilla luciferase. Cells were transfected in duplicated wells and these experiments were repeated three times.

ChIP assays. A chromatin immunoprecipitation (ChIP) assay was utilized to assess the sequences in the B7-H4 promoter which bind to p-Stat3. In brief, BV2 cells were fixed with formaldehyde. DNA was sheared to fragments of 200–1000 bp using sonication. The chromatin were incubated and precipitated with Ab against the p-Stat3 or IgG. The PCR was designed according to the data from UCSC (http://genome.ucsc.edu/). The primer sequences used are available upon request.

Phagocytosis Assay. After incubating for 72 hr with the experimental supernatant as described above, Mφs were transferred to a 12-well plate at a concentration of 1.0×10^5 cellswell^{-1} and cultured for 1 hr at 37°C in 95% air and 5% CO_2. Following cell adherence, fluorescent latex beads (Sigma, L3030, 2 mm) were added to wells and after 3 hr, the cells were washed to remove nonphagocytosed beads. Cells were then fixed in 4% paraformaldehyde and counter-stained with DAPI.

Cell Migration Assay. Cell migration assays were conducted using a 6.5mm Transwell®

with 8.0μm Pore Polycarbonate Membrane inserts as instructed (Product #3422). The well inserts were seeded with CD11b+ human macrophages or mouse microglia at a density of 2.0×10^4 and the lower chamber was loaded with control medium, CD133+ supernatant, CD133- supernatant or GL261 cells. The order of the two mouse cell lines was reversed in another plate. After overnight culture, all the cells on the upper surface of the membrane were removed and the migratory ones on the lower surface were fixed with 100% methanol and stained with 1% crystal violet (Sigma-Aldrich) before counting under a Nikon microscope. Measurements were presented as chemotaxis index following normalization to the total cell number.

C57BL/6 xenografttumor mouse model. C57BL/6 mice (males-only; age 6-8wks) were purchased from Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences, Experimental Animal Center. All mouse experiments were performed in compliance with Institutional Animal Care and Use Committee (IACUC) protocols. Before tumor implantation mice were anesthetized by intraperitoneal injection of 4% chloral hydrate solution (delivered at 10ml/kg). GL261 cells (CD133- group and enriched CD133+ cells group; 1×10^7 cells/ml) were harvested and resuspended in PBS on ice and were injected intracranially and stereotactically at 1×10^5 cells per mouse brain. The injection coordinates were 2.2mm lateral and 0.5mm posterior of the bregma and 3mm deep to the cortical surface of the brain. The inoculated mice were kept in the animal facility and sacrificed if the tumor burden posed an imminent threat, as outlined in the animal care guidelines. Moribund mice were anesthetized with a 4% chloral hydrate and perfused. Tumor formation was confirmed by H&E stain.

Human GBM cell line, U87MG, xenograft tumor model and tumor regression assay. For the subcutaneous glioma model, $1x10^7$ U87MG cells in 100μl PBS were injected into the right flank of SD mice (5-7wk old, one tumor per mouse). After 12d, 6×10^6 TAA-specific T cells together with 3×10^6 cells of (1) normal Mφs, (2) Mφs conditioned by CD133+ cells, (3) Mφs conditioned with CD133+ cells and transfected with sh-mock or (4) Mφs conditioned CD133+ cells and transfected with sh-B7-H4. These cells were injected in 100μl PBS into the caudal vein. Tumor growth was monitored twice weekly using a vernier caliper and tumor volume was calculated according to three perpendicular measurements. Tumor excision in sacrificed mice occurred after 15d. For the intracranial

tumor model, $1x10^6$ U87MG cells in 50μl PBS were implanted into the left striatum of SD mice (5-7wk old, one tumor per mouse). After 7d, PBS or 6×10^6 TAA-specific T cells mixed with 3×10^6 CD133+ cells conditioned by Mφs transfected with sh-mock or sh-B7-H4 were injected into the caudal vein. MRI scans of brains were performed on Day 7 and Day 21 post-inoculation. Mouse survival times were recorded and used for the Kaplan-Meier analysis.

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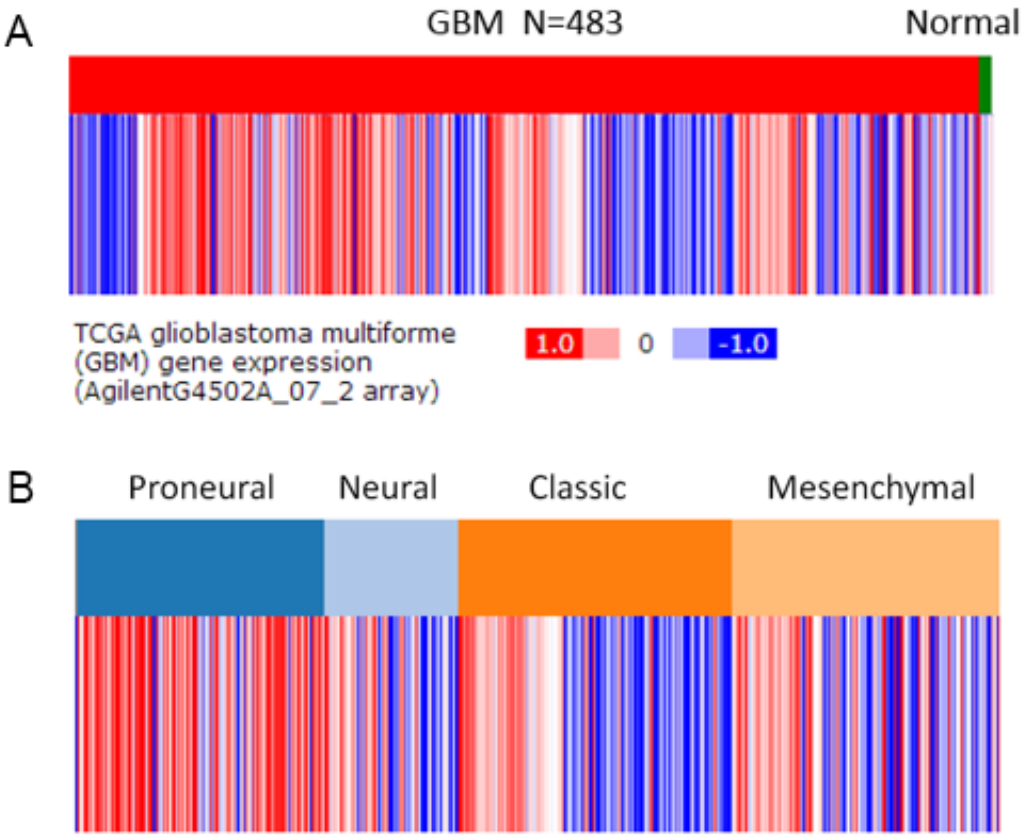
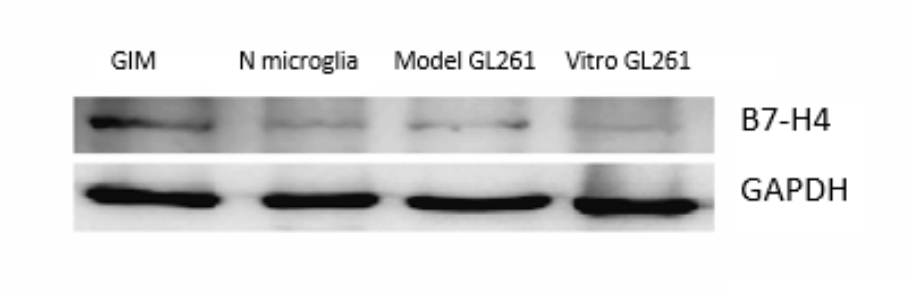


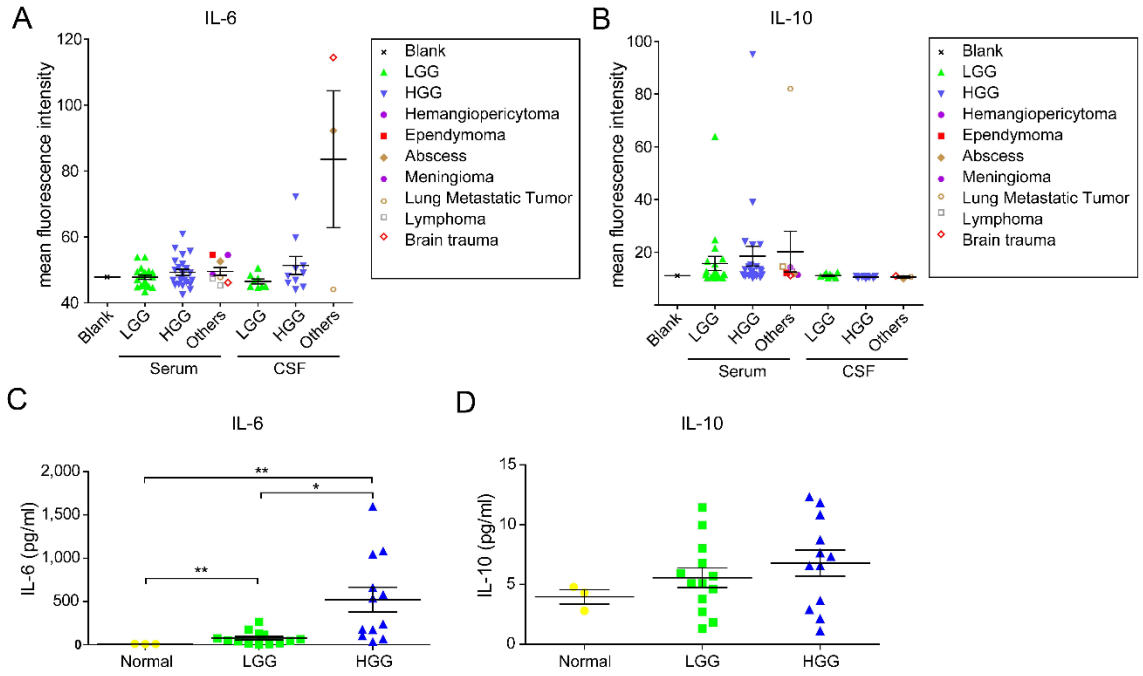
Supplementary Figure 1. Expression of B7-H4 in GBM from TCGA database.

(A) Heat maps of B7-H4 in GBM samples from the TCGA database (n=483) and in normal brain tissue (n=10). (B) Heat maps of B7-H4 in 4 phenotypes of GBM samples.



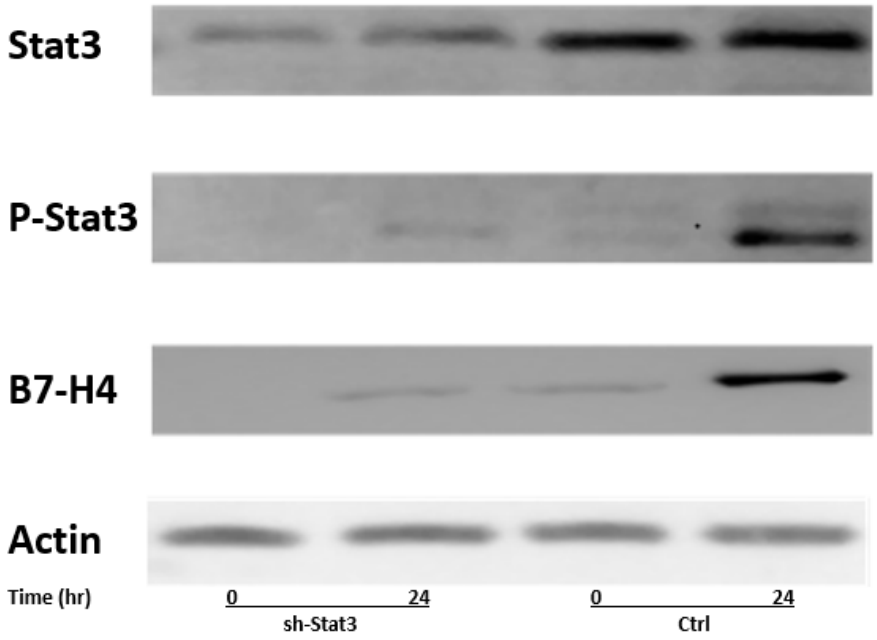
Supplementary Figure 2. WB analysis of GIMs, normal microglia, Model GL261 and Vitro GL261

WB analysis of B7-H4 in GIMs (20d after intracerebral injection of 1×10^5 GL261 cells per brain), normal microglia, Model GL261 (20d after intracerebral injection of 1×10^5 GL261 cells per brain). GIMs or normal microglia cells were isolated by percoll density separation and CD11b microbeads respectively from model and control.



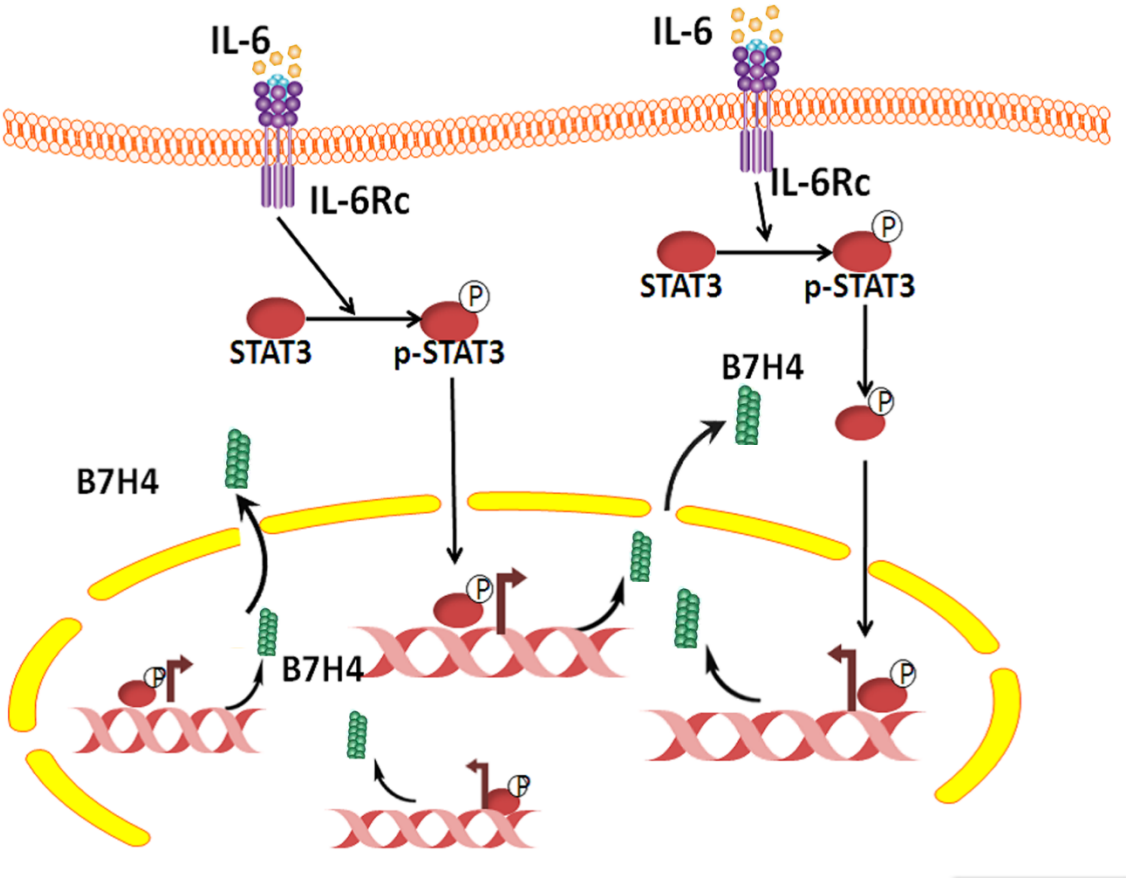
Supplementary Figure 3. IL-6 and IL-10 expression pattern in serum, CSF, and the glioma microenvironment.

(**A** and **B)** MFI (mean fluorescence index) of IL-6 and IL-10 detected by Flowcytomix (Blood: n=1/blank, 20/LGG, 23/HGG, 9/others. CSF: 8/LGG, 10/HGG, 3/others （intracranial diseases other than glioma）). (**C** and **D**) ELISA analysis of IL-6 or IL-10 concentration in tissue homogenates (n=3/normal, 13/LGG, 12/HGG). Error bars represent SEM. T test, *, $P < 0.05$; **, $P < 0.01$.



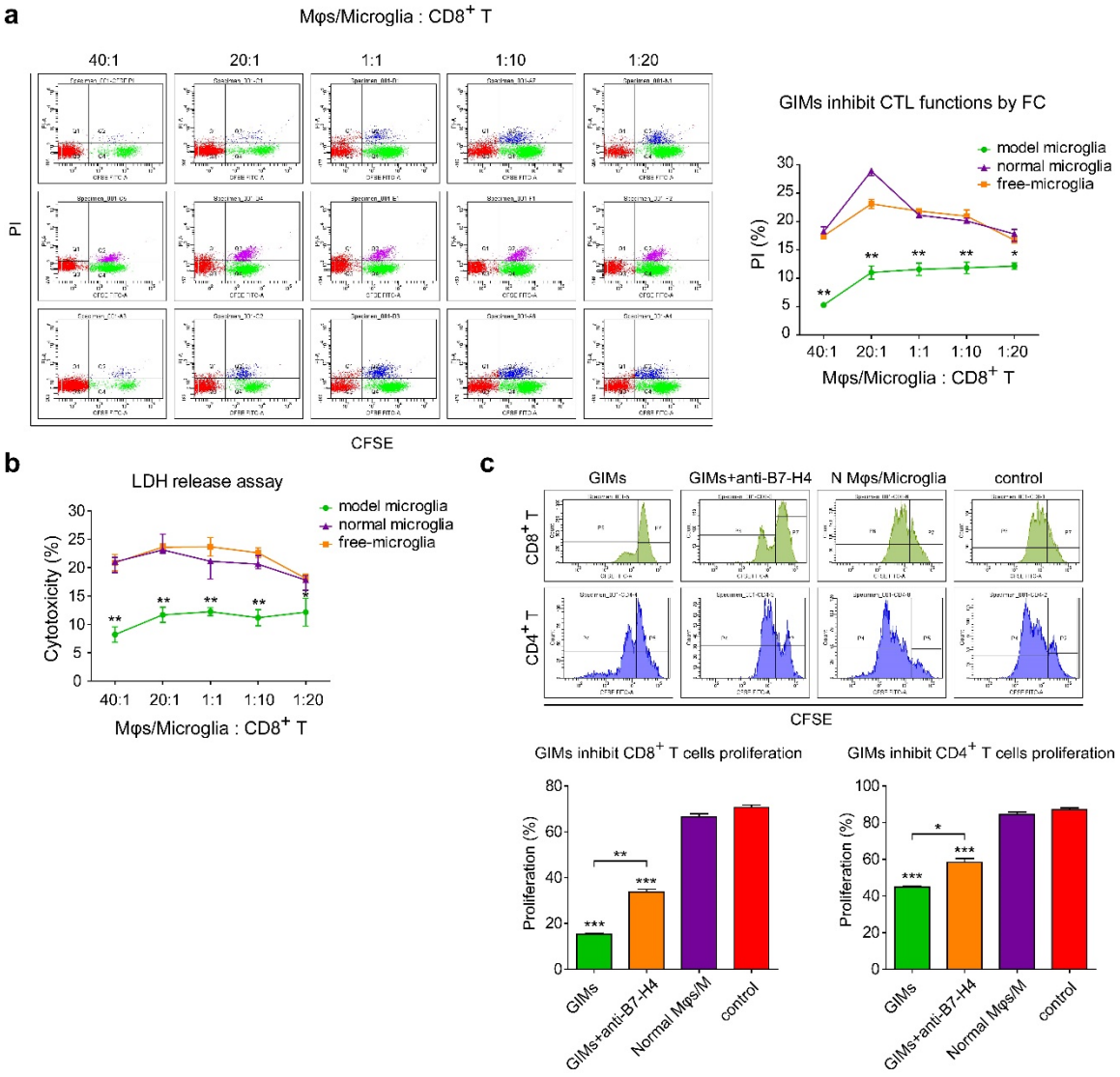
Supplementary Figure 4. WB analysis of BV2 microglia stimulated with CD133+ supernatant

The protein expressions of phosphorylated Stat3 (P-Stat3) and B7-H4 in BV2 microglia cells infected with shRNA3 or control after being stimulated with CD133+supernatant. ShRNA3 significantly inhibited P-Stat3 and B7-H4 protein expression after being stimulated with CD133+supernatant for 24hr.



Supplementary Figure 5. IL-6/Stat3/B7-H4 signal transduction pathway.

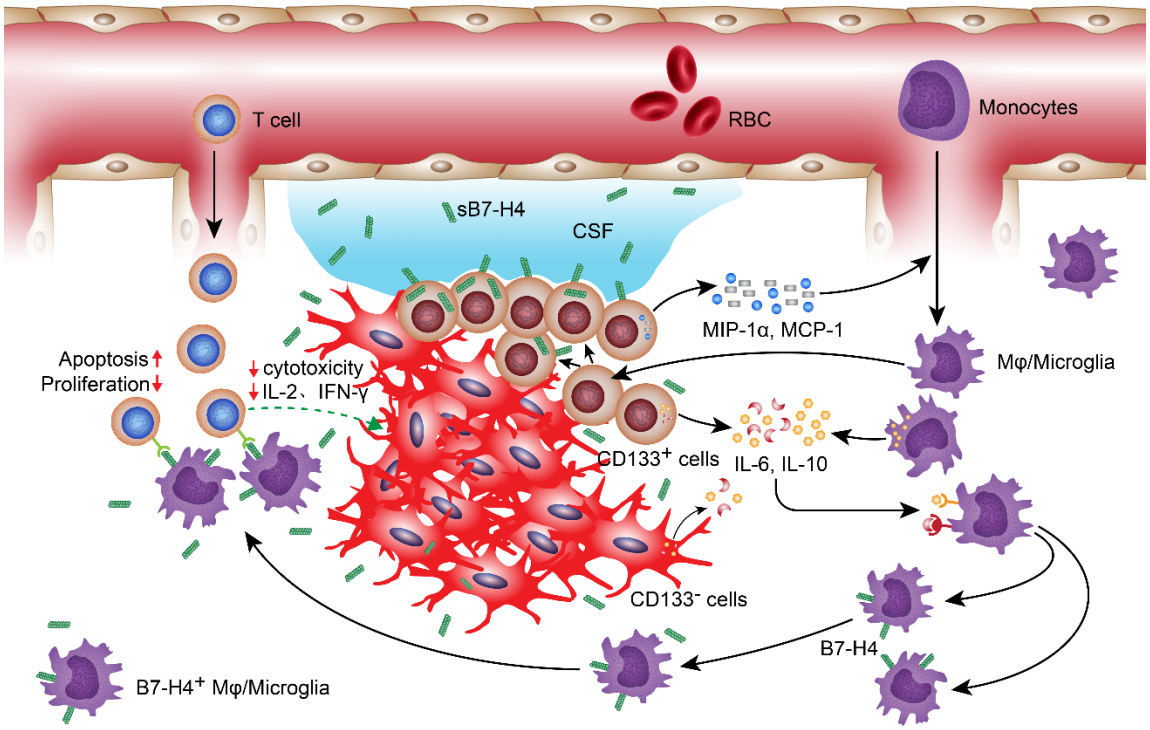
STAT3 is phosphorylated at either a tyrosine or a serine in response to the activation of the cell surface IL-6 receptors by IL-6. STAT3 then dimerizes and localizes to the nucleus, where it binds to the promoter region of the B7H4 target gene. As a result, the transcription of B7-H4 gene begins and the expression of B7H4 protein is up-regulated.



Supplementary Figure 6. GIMs enriched from CD133+ cells induced inhibition of T cell function via B7-H4 in vitro.

(**A**, left) CD8+ T cells from mouse spleens and mature DCs were grouped with CFSE-labeled GL261 cells (CD8+T: GL261, 1:1) and added to expansions of GIMs or normal Mφs/microglia (Mφs/microglia: CD8+T, 40:1; 20:1; 10:1; 1:1; 1:10; 1:20). (Right) FACS-generated dot plots in CFSE+ gate of GL261-cells labeled with propidium iodide reflected Mφs/microglia-mediated inhibition ability, as cytotoxicity at 12 hrs. Comparisons of PI% between GL261-cells cocultured with normal Mφs/microglia cells or GIMs were made for three separate experiments, using free-Mφs/microglia as a control

(mean±SEM, n=6; T test, *, $P<0.05$; **, $P<0.01$). (**B**) Cocultures of CD8+ T cells and mature DC were grouped with GIMs or normal Mφs/microglia (Mφs/microglia: CD8+ T, 40:1; 20:1; 10:1; 1:1; 1:10; 1:20) and expanded with or without GL261 cells present. Supernatants collected at 12hrs were analyzed by CytoTox 96® Non-Radioactive Assay kits (λ=490nm) and LDH release assays, demonstrating GIM-mediated inhibition of cytoxicity in CD8+T-cells in three separate experiments (mean±SEM, n=6; T test, **, $P<0.05$; **, $P<0.01$). (**C**,Top) CFSE-labeled CD8+ T and CD4+ T-cells were sorted, seeded into CD3-coated plates (10ng/ml), and incubated in RPMI 1640 complete medium supplemented with anti-CD28 (10ng/ml). Next, normal Mφs/microglia or GIMs incubated with and without anti-B7-H4 (10ng/ml) were added (1:1), using anti-CD3 and anti-CD28 in wells as positive controls. At 72 hr, cocultured cells were collected and analyzed by FACS to generate a representative histogram of CD8+ T cell proliferation across the different groups (left, bottom). CD4+ T cell proliferation was also quantified (right, bottom). Numbers reflect results collected from three independent experiments (mean±SEM, CD8+, n=6: T test, **, $P<0.01$; ***, $P<0.001$; CD4+, n=6: T test, *, $P<0.05$; ***, $P<0.001$).



Supplementary Figure 7. Outline for the increase of B7-H4 expression by immunosuppressive cross talk between CD133+ cells and GIMs. CD133+ cells in the tumor niche produce more chemokines such as MIP-1α、MCP-1 to recruit monocytes from the blood into the tumor microenvironment. CD133+ cells further induce monocytes-derived GIMs express B7-H4 via IL-6 and IL-10. GIMs stimulated CD133+ cell expression of B7-H4 and auto-regulation through IL-6. B7-H4+ Mφs/microglia convey suppressive signal to T cells, thus accelerating their apoptosis, reducing inflammatory cytokine production and attenuating cytotoxicity towards tumor cells. The level of sB7-H4 in CSF correlated with glioma malignancy grade.

Supplementary Table 1. Clinical characteristics of the patients for serum, CSF and tissue

Variable	Group for serum	Group for CSF	Group for tissue	Group for TMA
Number of patients	69	52	57	144
Age (yr)				
Median	45	44	44	45
Range	18-77	18-70	18-77	1-78
Gender				
Male	44	32	34	80
Female	25	20	23	64
Type of disease				
Glioma	63	48	57	138
Lymphoma	2	0	0	0
Metastatic tumor	1	2	0	0
Meningioma	1	1	0	0
Hemingiopericyoma	1	0	0	0
Abscess	1	1	0	3*
Trauma	0	0	0	3*
WHO Classification of Glioma				
I	3	3	2	11
II	29	13	21	22
III	15	17	17	35
IV	16	15	17	70

*surrounding edema tissue as control.

Supplementary Table 2. Clinical characteristics of the new diagnosed GBM patients for survival analysis

Variable	GBM group
Number of patients	70
Age (yr)	
Median	54
Range	29-78
Gender	
Male	39
Female	31
Position of tumor	
Frontal	22
Parietal	6
Temporal	14
Occipital	3
basal ganglia	3
corpus callosum	1
Thalamus	1
Multiple lobes	20
Extent of resection	
Total	57
Subtotal	13
Radiotherapy	
Yes	70
No	0
Chemotherapy（temozolomide）	
Yes	70
No	0
Median Survival (month)	18

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Co-stimulate or Co-inhibit Regulatory T Cells, Which Side to Go?

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ABSTRACT

Co-stimulatory and co-inhibitory molecules direct the "second signal," which largely determines the outcome of the "first signal" generated by the interaction of T cell receptor (TCR) with cognate MHC–peptide complex. The co-stimulatory and co-inhibitory signals are key mechanistic contributors to the regulation of adaptive immunity, especially the T cell–mediated immune response. Regulatory T cells (Tregs) are a special population of T cells, which unlike other T cells function as "attenuators" to suppress T cell immunity. Dysregulation of either the "second signal" or Tregs leads to an unbalanced immune system, which can result in a range of immune-related disorders, including autoimmune diseases, chronic infections, and tumors. In contrast, precise manipulation of these two systems offers tremendous clinical opportunities to treat these same diseases. Co-stimulatory and co-inhibitory molecules modulate immunity at molecular level, whereas Tregs delicately control the immune response at cellular level. Accumulating evidence has demonstrated that these two regulatory strategies converge and synergize with each other. This review discusses recent progress on the roles of co-stimulatory and co-inhibitory signals in the context of Tregs.

KEYWORDS

Co-inhibition; co-stimulation; immunotherapy; Treg

Introduction

Co-stimulation and co-inhibition

Co-stimulatory and co-inhibitory molecules are pivotal cell-surface proteins, largely composed of members of the immunoglobulin superfamily (IgSF) and tumor necrosis factor/receptor superfamily (TNFSF/TNFRSF) (Chen & Flies, 2013). These molecules are vital for T cell activation and subsequent immune responses, as they provide the secondary signal that determines the course, duration, and extent of the response following the initial signal provided by engagement of the T cell receptor (TCR) and the major histocompatibility complex (MHC)–peptide complex. The co-stimulatory receptors transduce positive signals to facilitate or amplify the adaptive immune response, whereas the co-inhibitory receptors produce negative signals to attenuate the T cell response (Zang & Allison, 2007).

The CD28:B7 family members are among the most extensively studied co-stimulatory and co-inhibitory molecules. The CD28:B7 family belongs to the IgSF, the members of

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which all share similar overall structural features, with each Ig domain formed by two mixed beta-sheets (Chattopadhyay et al., 2009). The CD28:B7 family contains the first identified co-stimulatory receptor CD28, co-inhibitory receptor CTLA-4, and their joint ligands B7-1 and B7-2 (Zang & Allison, 2007). Activation of the CD28 receptor by its ligands, or agonistic antibodies, was shown to prevent T cell energy induction and promote T cell proliferation and cytokine IL-2 production, thus establishing CD28 as a central co-stimulatory receptor (Gimmi et al., 1991; Harding et al., 1992; Koulova et al., 1991; Linsley et al., 1991). In contrast to CD28, which is constitutively expressed on T cell surface and enhance T cell activity, CTLA-4 is induced following T cell activation and serves as a co-inhibitory receptor to suppress T cell response (Rudd et al., 2009). The CD28/CTLA-4 and B7-1/B7-2 pathways were the first characterized co-stimulatory and co-inhibitory pathways, and have been under intense study since their discovery. PD-1 and PD-L1/PD-L2 pathways were later reported to be another co-inhibitory pathways to inhibit the T cell–mediated immunity (Freeman et al., 2000).

Working in concert with the CD28:B7 family of the IgSF, additional co-stimulatory and co-inhibitory molecules are represented by members of the TNFSF/TNFRSF. Most of the TNF ligands form a homotrimeric assembly, with each monomer adopting the typical "jelly-roll" fold involving two parallel β-sheets. TNF receptors possess ectodomains characterized by varying numbers of tandemly linked cysteine-rich domains (CRDs) (Chattopadhyay et al., 2009). In most circumstances, one TNF ligand is able to bind three TNF receptors through the grooves between each protomer (Chattopadhyay et al., 2009). The engagement of TNF receptors with TNF ligands leads to the trimerization, in some cases dimerization, of TNF receptors and activates the intracellular signal transduction pathways involving the assembly of intracellular scaffolding and signaling complexes.

The importance of co-stimulatory and co-inhibitory molecules in regulating the immune system has been demonstrated by the successful development and clinical application of drugs targeting these molecules. Ipilimumab (Yervoy, Bristol-Myers Squibb, USA) is an FDA-approved function-blocking monoclonal antibody (mAb) that specifically targets CTLA-4 to inhibit the associated co-inhibitory signal, resulting in a systemic enhancement of T cell activity. Ipilimumab represents the first clinical treatment to significantly prolong survival in late-stage melanoma cancer patients and marks a milestone for cancer immunotherapy (Chodon et al., 2015; Zang and Allison, 2007). Selective inhibition of the PD-1/PD-L1 inhibitory pathway by mAbs has also resulted in two FDA-approved drugs pembrolizumab (Keytruda, Merck & Co., USA) and nivolumab (Opdivo, Bristol-Myers Squibb, USA) to treat cancers (Chinai et al., 2015).

Despite the enormous success on targeting these proteins to treat immune-related diseases, it has been gradually realized that the outcomes of these co-stimulatory and co-inhibitory molecules are not as straightforward as what they were originally discovered. Given the immense diversity of the cellular expression, structures, and their interaction networks, these molecules may have totally different outcomes on the adaptive immunity. For example, expression of the co-stimulatory receptor CD28 on conventional effector T cells (Teffs) upregulates immune response. However, expression of CD28 on a special suppressive T cell population, termed regulatory T cells (Tregs), promotes immune inhibition and grants some levels of immune tolerance. As more of the co-stimulatory and co-inhibitory molecules are emerging, more thorough understanding of how these

molecules affect the immunity, especially in the context of Tregs, is required for the precise manipulation of the immune system through co-stimulation and co-inhibition.

Tregs

Tregs are a T cell subpopulation produced by the normal immune system, which provide suppressive signals to prevent overly aggressive immune responses. Tregs naturally arise within the thymus as a functionally mature T cell lineage and can also be induced in the periphery from naive T cells (Sakaguchi et al., 2008). Both thymus-derived natural Tregs (tTregs) and peripherally induced Tregs (pTregs) participate in controlling the magnitude of the immunity. Depletion of Tregs can lead to the development of a range of autoimmune conditions, including colitis, which possibly result from lack of control of bacteria-driven inflammatory responses in the mucosal system (Singh et al., 2001). Conversely, elimination or reduction of Tregs can overcome the immunosuppressive mechanisms utilized by tumors or chronically infectious microbes to evade the host immune system, and provides a strategy for the eradication of tumors or microbes (Belkaid & Rouse, 2005; Wollenberg et al., 2011). Neonatal thymectomy experiments in mice cause autoimmune disease, demonstrating thymus-derived tTregs are key to immune tolerance, and also demonstrate that peripheral pTregs are not sufficient to suppress auto-reactive immunity (Asano et al., 1996; Bonomo et al., 1995; Sakaguchi et al., 1995). However, peripheral pTregs do make important contributions to controlling autoimmune responses (Haribhai et al., 2011; Josefowicz et al., 2012; Samstein et al., 2012), as pTreg deficiency is sufficient to evoke T cell–mediated autoimmune conditions (Yadav et al., 2013). In contrast, enrichment of pTregs in mice ameliorates allergy, builds immunological tolerance to transplanted organs, and enhances feto-maternal tolerance (Sakaguchi, 2005).

Tregs are characterized by the expression of cell-surface receptor CD25 and transcription factor Foxp3. CD25 is the α chain of the heterotrimeric IL-2 receptor complex, which captures IL-2 with high affinity. CD25 is critical for Treg function, as mice deficient in CD25 develop lymphoproliferative autoimmune disease and are hyperreactive to commensal microbiota, with a phenotype resembling that of pTreg knockout mice (Sakaguchi et al., 2008). The transcription factor Foxp3 programs the development and function of Tregs. In humans, mutations in the FOXP3 gene results in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett et al., 2001). Similarly, Foxp3-null mice or the mouse strain Scurfy, which is defective in Foxp3 gene, develop deleterious hyperreactive immunological phenotypes resembling IPEX (Fontenot et al., 2003). Overexpression of Foxp3 in transgenic mice increases the number of Tregs and delays the catastrophic disease in CTLA-4 $^{-/-}$ mice (Khattri et al., 2003). Ectopic expression of Foxp3 in naive T cells upregulates the expression of CD25, the co-stimulatory molecule GITR (glucocorticoid-induced tumor necrosis factor receptor related protein) and co-inhibitory molecule CTLA-4, and programs the expression of other Treg functional molecules (Fontenot et al., 2003).

Several mechanisms have been proposed for Treg-mediated suppression. For example, Tregs may secrete immunosuppressive cytokines and absorb cytokines necessary for other T cells to proliferate and function. TGF-β secreted by Tregs can mediate suppression and program the Teffs to be more susceptible to suppression (von Boehmer, 2005).

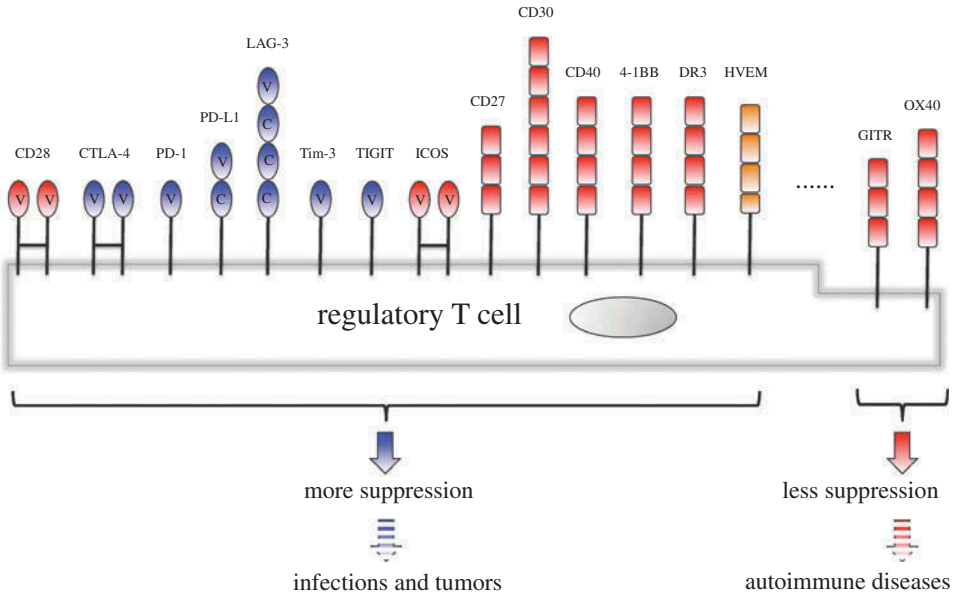

Figure 1. The co-stimulatory (red) and co-inhibitory (blue) molecules are classified as two groups based on their impact on Tregs. The left side shows the group of molecules that can enhance the suppression function of Tregs after stimulation. The right side shows the group of molecules that can impair the suppression function of Tregs after stimulation. Members of IgSF are represented as cognate numbers of Ig domains (*ovals*). "V" and "C" stand for IgV and IgC domains, respectively. Members of TNFRSF are represented as cognate numbers of CRDs (*rectangles*). HVEM is colored as orange as it can be either co-stimulatory or co-inhibitory, depending on different engaging ligands.

Competitive consumption of cytokines by Tregs, including IL-2, deprives Teffs of survival cytokines and induces apoptosis of these Teffs (Pandiyan et al., 2007). Tregs can also modify the function of, or even kill, antigen-presenting cells (APCs) in a direct cell contact–dependent manner (Sakaguchi et al., 2008). The co-stimulatory and co-inhibitory molecules expressed by both Tregs and APCs play essential roles in the development and suppressive functions of Tregs at multiple steps (Bour-Jordan & Bluestone, 2009). In this review, we discuss the roles of several co-stimulatory and co-inhibitory molecules in different aspects of Treg function (Figure 1) and explore the potential of cancer immunotherapies targeting co-stimulatory, co-inhibitory signal, and Tregs.

CD28

CD28, the prototypical co-stimulatory receptor, is constitutively expressed on almost all human $CD4^+$ T cells and on about half of human $CD8^+$ T cells, whereas it is expressed on almost all matured $CD4^+$ and $CD8^+$ T cells in mouse (Acuto & Michel, 2003). CD28 exists as a disulfide-linked homodimer on the cell surface, with each monomer composed of one single immunoglobulin variable (IgV) domain. The CD28 ligands, B7-1 and B7-2, are each composed of a membrane distal IgV domain and a membrane proximal immunoglobulin

constant (IgC) domain (Chattopadhyay et al., 2009). The interactions of CD28 with B7-1 and B7-2, involving their respective IgV domains, are involved in a wide range of T cell functions, including T cell proliferation, cytokine production, survival, and T cell–dependent antibody responses (Lenschow et al., 1996; Salomon & Bluestone, 2001). It seemed with clear evidence that CD28 and B7-1/B7-2 pathway is the most prominent driving force of positive immune response. However, surprisingly, CD28 or B7-1/B7-2 knockout mice, whereas in non-autoimmune backgrounds and many antigen-induced autoimmune backgrounds, exhibited significant immune-deficient phenotypes, and exacerbated the autoimmune conditions when bred into the non-obese diabetic (NOD) background (Bour-Jordan and Bluestone, 2009; Lenschow et al., 1996; Salomon et al., 2000). In combination with the vast Treg literature, these results for the first time suggested a role for CD28 in T cell suppressor function. It is now well established that though CD28 transduces co-stimulatory signal to Teffs, it is also required for the homeostasis and function of the suppressing Tregs.

In NOD mice with either CD28 or B7-1/B7-2 knockout, the incidence and progression of spontaneous autoimmune diabetes were enhanced. Moreover, these mice developed other autoimmune disorders such as autoimmune exocrine pancreatitis, and displayed higher degree of lymphocyte penetration and more severe diabetes compared with the controls (Lenschow et al., 1996; Meagher et al., 2008; Salomon et al., 2000). Further assessment of B7 knockout NOD mice revealed that the immunoregulatory $CD4^+CD25^+$ Tregs were absent. Injection of the wildtype $CD4^+CD25^+$ Tregs into CD28 knockout NOD mice restored the control of diabetic disease (Salomon et al., 2000; Tang et al., 2004). Additionally, blockade of CD28:B7 pathway dramatically decreases the Tregs population in all other normal and autoimmune strains (Bour-Jordan and Bluestone, 2009; Gogishvili et al., 2013; Sansom and Walker, 2006). Disruption of CD28 signaling using CTLA-4-Ig or antagonistic mAbs against B7 ligands induced a rapid decrease in the number of $CD4^+CD25^+$ Tregs within 9 days of treatment (Salomon et al., 2000). The dramatic decrease on Tregs after treatment is similar to that observed in thymectomized mice. However, thymectomized mice did not affect the Tregs within 10 days, suggesting that impairment of the CD28:B7 pathway has a direct impact on pTregs, and the CD28:B7 pathway is required for the maintenance of pTregs (Tang et al., 2003). Furthermore, transplanted Tregs exhibited similar decreases when B7-specific blocking reagents were utilized (Bour-Jordan and Bluestone, 2009). These results suggest that the CD28:B7 pathway is critical for the homeostasis of both thymus-derived tTregs and peripheral pTregs, and may leverage the threshold of the autoimmune diseases by modulating the homeostasis of Tregs.

CD28 acts in concert with the signal provided by TCR engagement to promote T cell activation and proliferation, largely by accelerating T cell division, as well as boosting cytokine production (Gett and Hodgkin, 2000; Sansom and Walker, 2006; Thompson et al., 1989). While CD28-associated signaling promotes proliferation of both Teffs and Tregs *in vitro, in vivo* studies suggest CD28 has a more profound effect on Treg proliferation (Hori et al., 2002). Several other experiments produced consistent results, which all demonstrated the rapid proliferation of Tregs *in vivo* (Fisson et al., 2003; Tang et al., 2003; Walker et al., 2003). Abrogation of CD28:B7 engagement by antagonistic anti-B7 mAbs or in B7 ligand knockout mice recipients completely prevented Treg expansion *in vivo* in a transfer model (Tang et al., 2003). Conversely, a "superagonistic" anti-CD28 mAb

preferentially expanded Tregs over other T cell subsets *in vivo*, resulting in a 20-fold Tregs expansion within 3 days after a single mAb treatment (Lin & Hünig, 2003). Administration of very low dosages of CD28 superagonist to rats dramatically enhanced the proliferation of suppressive Tregs; but, not the other T cells, and afforded protection from experimental autoimmune encephalomyelitis (EAE) (Beyersdorf et al., 2005). However, intravenous administration of agonistic anti-CD28 mAbs (working name TGN1412) even at sub-clinical dose induced rapid cytokine storms and lead to catastrophic systemic organ failures in patients of a phase 1 clinical trial (Suntharalingam et al., 2006).

CD28 enhances the survival of T cells by activating phosphatidylinositol 3-kinase (PI3K) and subsequent AKT kinase (Sansom & Walker, 2006). CD28 activation also renders T cells more resistant to apoptosis by upregulation of the prosurvival protein Bcl-x_L (Okkenhaug et al., 2001; Wu et al., 2005). As CD28 induces IL-2 secretion, CD28 may indirectly promote Treg survival by upregulating IL-2 production (Bour-Jordan and Bluestone, 2009). The role of CD28 in Treg functions has been rarely reported, largely because CD28 is required for the homeostasis of Tregs. Several recent studies using Tregs-specific *Cd28* conditional knockout mice revealed a cell-intrinsic function for CD28 in Tregs. Although the *Cd28* conditional knockout mice presented normal numbers of $Foxp3^+$ cells, the animals still developed severe autoimmune conditions, indicating CD28 is indispensable for the immunoregulatory function of Tregs (Zhang et al., 2013, 2015).

CTLA-4

CTLA-4, the co-inhibitory counterpart of CD28, also engages the B7-1 and B7-2 ligands, albeit with 10- and 100-fold higher affinities toward B7-1 and B7-2, respectively (Collins et al., 2002). Like CD28, CTLA-4 is composed of a single extracellular IgV domain followed by a stalk region, a transmembrane domain, and a cytoplasmic tail. Two CTLA-4 extracellular stalk regions share a disulfide bond, which brings together two CTLA-4 to form a covalently linked homodimer (Chattopadhyay et al., 2009). In contrast to CD28, CTLA-4 expression is induced subsequent to T cell activation (Teft et al., 2006). Tregs are notable exceptions as they constitutively express CTLA-4 (Takahashi et al., 2000). The transcription factor Foxp3 has been demonstrated to upregulate CTLA-4 expression on Tregs (Sansom and Walker, 2006). Evidence for the convergence of CTLA-4 and Treg-mediated tolerance comes from the CTLA-4 knockout mice and Foxp3 knockout mice. Deficiency of CTLA-4 or Foxp3 elicits similar catastrophic autoimmune phenotypes, suggesting potential links between the CTLA-4 pathway and Treg function (Walker, 2013).

Initial reports suggested that mAb-mediated blockade of CTLA-4 resulted in loss of the suppressive functions of Tregs (Read, et al., 2000; Takahashi et al., 2000). Another possibility was that the observations were the consequence of augmented conventional T cell activity (Tconvs) due to loss of inhibitory CTLA-4 signaling (Thornton et al., 2004). In several *in vitro* Treg suppression assays, absence of the CTLA-4 signal also abrogated Treg function (Tai et al., 2012; Wing et al., 2008). The most compelling evidence in support of a role for CTLA-4 in Treg function comes from *in vivo* experiments, in which the CTLA-4 signal was specifically manipulated in Tregs. In a T cell–mediated colitis

model, administration of wildtype $CD4^+CD25^+$ Tregs was able to suppress the colitis, whereas administrations of either B7-1/B7-2/CTLA-4 knockout $CD4^+CD25^+$ Tregs or wildtype $CD4^+CD25^+$ Tregs in combination with antagonistic anti-CTLA-4 mAbs did not protect the mice from colitis (Read et al., 2006). Specific deletion of the CTLA-4 gene in Tregs impaired Treg-mediated suppression and resulted in hyper-elevated T cell–mediated immunity, including lymphadenopathy, splenomegaly, and lymphocyte tissue infiltration (Wing et al., 2008). Many additional *in vivo* experiments demonstrated that Tregs with impaired CTLA-4 function failed to control the autoimmune responses in various autoimmune-prone experimental settings (Friedline et al., 2009; Ise et al., 2010; Jain et al., 2010; Schmidt et al., 2009; Walker, 2013).

CTLA-4 may exert its function on Tregs by modifying APC behavior. *In vitro* imaging studies identified Tregs aggregated around APCs (Onishi et al., 2008; Tang & Krummel, 2006). The persistent Treg–APC contacts in lymph nodes *in situ* are important for suppressing the T cell immunity, as demonstrated by the significant attenuation of the ability of these APCs to activate Teffs. There were no stable direct contacts between Tregs and $CD4^+CD25^-$ T helper cells (T_H cells), suggesting APCs are central for Treg function (Tang et al., 2006). In a CTLA-4-dependent manner, Tregs could induce expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO), and direct downregulation of B7 ligands on APCs, which are shared by co-stimulatory receptor CD28, probably by uptake of the ligands through trogocytosis (Bour-Jordan & Bluestone, 2009; Cederbom et al., 2000; Fallarino et al., 2003; Sprent, 2005).

It is now well established that CTLA-4 plays a significant role in Treg-mediated suppression of immunity. However, it is important to note reports that CTLA-4-deficient Tregs still possess suppressive function both *in vivo* and *in vitro* in different experimental settings (Stumpf et al., 2013; Verhagen et al., 2009; Walker, 2013). Although CTLA-4 is required for the function of Tregs, alternative compensatory mechanisms may exist in some circumstances (Wing et al., 2011). For example, depletion of CTLA-4 may upregulate other suppressive signaling molecules on Tregs, thus compensating/substituting for CTLA-4 in Treg function (Paterson et al., 2015).

In contrast to the critical role of CD28 in Tregs development and survival, CTLA-4 does not appear to be crucial for the generation and survival of Tregs, as Treg-specific deficiency of CTLA-4 did not show any deficit in Tregs development, expansion, and survival in a CTLA-4 Treg-intrinsic manner in non-inflammatory conditions (Bour-Jordan & Bluestone, 2009). However, there are also reports showing CTLA-4-deficient mice or administration of antagonistic anti-CTLA-4 mAb result in an amplified population of $CD4^+CD25^+$ Tregs, suggesting CTLA-4 may serve as a negative feedback loop to limit the population size of Tregs, most likely in a Treg-extrinsic manner (Bour-Jordan & Bluestone, 2009; Paterson et al., 2015; Schmidt et al., 2009; Tang et al., 2008).

PD-1/PD-L1 axis

The co-inhibitory receptor PD-1 belongs to the IgSF, and possesses a single IgV ectodomain and an intracellular domain containing two signaling motifs (Ishida et al., 1992). PD-1 has two ligands, PD-L1 and PD-L2, which both have a sequential IgV and IgC domains, and a cytoplasmic tail (Riella et al., 2012). It was recently reported that PD-L1 also interacts with B7-1, resulting in an inhibitory signal (Butte et al., 2007). Chemical

crosslinking experiments revealed that the interface of PD-L1/B7-1 overlaps with the interfaces of CTLA-4/B7-1 and PD-1/PD-L1, indicating they can possibly compete with each other (Butte et al., 2007). PD-L2 also interacts with RGMb (repulsive guidance molecule b) to attenuate respiratory immunity (Xiao et al., 2014). PD-1 expression is induced after activation of T cells, B cells, natural killer (NK) cells, and other APCs, including monocytes and myeloid dendritic cells (DCs) (Keir et al., 2008). PD-L1 has a broad expression profile, which includes most hematopoietic cells and a wide range of non-hematopoietic cell constitutively expressing PD-L1 (Cederbom et al., 2000). PD-L2 has a more restricted expression profile, with only a group of APCs inducing to express PD-L2 (Cederbom et al., 2000). Both PD-1 and PD-L1 are highly expressed on $Foxp3^+$ Tregs (Francisco et al., 2010).

The PD-1/PD-L1 axis has been found to play a role in the generation of peripheral pTregs. PD-L1, but not PD-L2, was required for the TGF-β-dependent conversion of naive T cells to Tregs, as $PD\text{-}L1^{-/-}$ DCs but not $PD\text{-}L2^{-/-}$ DCs failed to convert naive T cell in an *in vitro* experimental setting (Wang et al., 2008). In addition, $PD\text{-}L1^{-/-}$ APCs and $PD\text{-}L1^{-/-}PD\text{-}L2^{-/-}$ APCs retained similar minimal ability to convert naive CD4 T cells to pTregs. Conversely, PD-L1-coated beads were able to induce pTregs *in vitro*, indicating PD-L1 is important for pTregs induction (Francisco et al., 2009). PD-L1 positive T cells or irradiated K562 myeloid tumor cells were able to convert T_H1 cells into Tregs *in vivo*, whereas inhibition of PD-1 expression on T_H1 or inhibition of PD-1 signaling by SHP1/2 inhibitor prevented conversion during PD-L1 challenge (Amarnath et al., 2011). Moreover, murine vascular endothelium could induce peripheral $CD4^+CD25^+$ Tregs in a PD-L1-dependent fashion (Krupnick et al., 2005). In the EAE mouse model, the increased frequency of Tregs can be abrogated by PD-1 deficiency (Wang et al., 2010). These results indicate that the PD-1/PD-L1 axis contributes to peripheral tolerance by inducing peripheral pTregs.

Interestingly, in an autoimmune-like graft-versus-host disease (GVHD) model, it was shown that the donor Tregs in the recipients were predominantly expanded from tTregs, with few originating from pTregs (Yi et al., 2011). In addition, B7-1 rather than PD-1 expressed by donor Tregs was demonstrated to augment the proliferation and survival of tTregs through ligation with PD-L1 expressed on host APCs (Yi et al., 2011). However, in HCV chronically infected patients, upregulation of PD-1 on Tregs was found to be associated with the relatively lower expansion of Tregs *in vivo*. Blockade of PD-1/PD-L1 or PD-L1/B7-1 by antagonistic antibodies improved the *in vitro* proliferation and function of Tregs derived from the livers of patients, indicating that PD-1/PD-L1-related processes temper Tregs' function in chronic HCV infection patients (Franceschini et al., 2009). In contrast, in a lymphocytic choriomeningitis virus (LCMV) chronic mouse model, upregulation of PD-1 on Tregs facilitated the expansion and increased the suppressive capacity of Tregs (Park et al., 2015). Direct contact of PD-1 on Tregs and PD-L1 on $CD8^+$ T cells was partially responsible for the observed T cell suppression (Park et al., 2015). Many questions remain to be addressed, including reconciliation of the debate on the role of PD-1/PD-L1 in the function of Tregs. Additional studies in different settings need to be conducted, ideally exploiting mouse models with specific conditional knockout of PD-1/PD-L1/B7-1 in Tregs.

GITR

The co-stimulatory receptor GITR is a member of TNFRSF, which engages with the TNFSF ligand GITRL. Both human and mouse GITR has three tandem CRDs followed by a stalk region, a trans-membrane domain, and a cytoplasmic domain. Human GITRL exhibits an atypical expanded homotrimeic assembly, whereas mouse GITRL possesses an even more unusual dimeric structure (Chattopadhyay et al., 2007, 2008). Notably, unlike most members of the TNF/TNFR families, the human and murine GITR and GITRL do not cross react due to these distinct structural properties. In humans, GITRL protein expression can be detected in non-lymphoid tissues, including vascular endothelial cells, but cannot be detected on different PBMC subsets (Tuyaerts et al., 2007). In contrast, mouse GITRL protein is constitutively expressed on APCs, including DCs, freshly isolated macrophages, and subsets of B cells (Shevach & Stephens, 2006). The GITR receptor is constitutively expressed at high levels on Tregs and at low levels on other $CD4^+$ and $CD8^+$ T cells. Upon TCR ligation, GITR is upregulated on $CD4^+$ and $CD8^+$ T cells, with peak expression occurring 24–72 hours after TCR activation (McHugh et al., 2002; Shevach & Stephens, 2006).

Administration of anti-GITR antibodies (polyclonal or agonistic monoclonal) in mice abrogated the immunological tolerance conferred by Tregs, demonstrating a functional role for GITR in regulating Treg-mediated tolerance (McHugh et al., 2002; Shimizu et al., 2002). In another study using mice with advanced tumors, a single treatment with agonist anti-GITR mAbs evoked effective tumor immunity, resulting in tumor eradication. Stimulation of GITR reduced the $Foxp3^+$ Tregs in tumors, hampered Treg-mediated suppression, and enhanced $CD4^+$ and $CD8^+$ effector T cells infiltration in tumors (Ko et al., 2005). Using combinations of wildtype and GITR-deficient mice, one study demonstrated that the increased resistance of Teffs to Tregs after GITR stimulation *in vitro* and *in vivo* was primarily due to GITR on Teffs rather than Tregs (Ephrem et al., 2013; Stephens et al., 2004). Indeed, stimulation of GITR by different kinds of agonist reagents augmented Teffs proliferation, cytokine production, and survival (Igarashi et al., 2008; Kanamaru et al., 2004). In addition, GITR-deficient Tregs were not compromised in their ability to inhibit T cell expansion *in vitro* (Ephrem et al., 2013; Stephens et al., 2004). Thus, GITR may primarily control the Treg-mediated suppression in a Treg-extrinsic and not a Treg-intrinsic manner.

Though GITR may not be essential for Treg function, GITR can promote the expansion of Tregs. $GITR^{-/-}$ mice harbored normal numbers of Tregs in the thymus compared with wildtype mice, but about 33% fewer $CD25^+CD4^+$ Tregs in the spleen and peripheral lymph nodes, indicating GITR may contribute to the homeostasis of peripheral Tregs (Ronchetti et al., 2004; Stephens et al., 2004). GITRL-Fc treatment resulted in a dramatic expansion of Tregs and a mild expansion of Tconvs in naive mice, though the increase of Tregs frequency was transient and the percentage of Tregs returned to normal after the treatment (Ephrem et al., 2013). In a B cell–specific GITRL transgenic mouse model, in which GITRL expression was driven by the CD19 promoter, both the numbers of $CD4^+$ Tregs and Teffs were increased due to increased proliferation (van Olffen et al., 2009). B cells could restore the numbers of Tregs in EAE mouse model through the expression of GITRL and maintain tolerance (Ray et al., 2012). Thus, GITR may play a role in self-tolerance by adjusting the relative populations of Tregs and Teffs.

OX40

OX40 is another co-stimulatory receptor belonging to the TNFRSF. The ectodomain of OX40 contains four tandem CRDs, which engage the TNF ligand OX40L. Similar to human GITRL, OX40L possesses an atypical expanded homotrimeric organization, which is able to interact with three OX40 through the grooves between each protomer (Compaan & Hymowitz, 2006). OX40L is predominantly expressed on APCs, including DCs, activated B cells, microglia, and vascular endothelial cells (Takeda et al., 2004; Watts, 2005). OX40 expression is induced in activated T cells, whereas it is constitutively expressed on Tregs (Piconese et al., 2010).

There is considerable evidence supporting the role of OX40 in impairing the suppressive function of Tregs. OX40L transgenic mice, which express OX40L on T cells, spontaneously developed IBD-like colitis, whereas blockade of OX40/OX40L interaction or transfer of $CD4^+CD25^+$ Tregs prevented the disease (Malmström et al., 2001; Murata et al., 2002; Read et al., 2000). Further studies demonstrated Teffs were more resistant to Tregs when exposed to OX40L cells or agonistic anti-OX40 mAbs (Takeda et al., 2004). In GVHD models, triggering of OX40 inhibited the suppressor function of Tregs, possibly by reducing Foxp3 gene expression (Valzasina et al., 2005; Vu et al., 2007). In the context of cancer immunotherapy, the use of agonist anti-OX40 mAbs alone or combined use of cyclophosphamide (CTX) and agonist anti-OX40 mAbs reduced the suppressive immunity imposed by Tregs in the tumor and elicited tumor clearance (Hirschhorn-Cymerman et al., 2009; Piconese et al., 2008). In addition, OX40 signaling was shown to inhibit the TGF-β and antigen-mediated conversion of naive CD4 T cell to Tregs (So & Croft, 2007).

In contrast, other studies identified OX40 as a critical factor in stimulating Treg proliferation. For example, in a colitis mouse model, OX40 was found preferentially expressed on intestinal T cells and promote the accumulation of $Foxp3^+$ Tregs in the colon and suppressed the colitis (Griseri et al., 2010). OX40 signaling also promoted the fitness of Tregs by optimizing the Tregs responsiveness to IL-2, resulting in the inhibition of lymphopenia-driven colitis (Piconese et al., 2010). Though the tumors were eradicated after combination therapy, expanded Tregs were observed in the periphery (Hirschhorn-Cymerman et al., 2009). Interestingly, it has been reported that in the presence of Th1/2 cytokines, OX40 stimulation could block TGF-β-mediated conversion of activated T cells to Tregs, whereas in the absence of IFN-γ and IL-4, OX40 signaling enhanced the accumulation of Tregs, suggesting the cytokine milieu may be a key consideration for reconciling these disparate results (Ruby et al., 2009).

Other co-stimulatory and co-inhibitory molecules

Accumulating evidence supports the view that most co-stimulatory and co-inhibitory molecules, including members from both IgSF and TNFSF/TNFRSF, impact on Treg function, either primarily or secondarily to their effects on Tconvs (Figure 1). The co-inhibitory molecule LAG-3 (lymphocyte activation gene-3), which is constitutively expressed on Tregs, plays a crucial role in suppressing Teffs, possibly by engaging the MHC-II expressed DCs and inhibiting DCs activation (Huang et al., 2004; Liang et al., 2008). LAG-3 defines a population of active $CD4^+CD25^+Foxp3^+$ Tregs, which is expanded at tumor sites in cancer patients (Camisaschi et al., 2010). Though the

receptor for co-inhibitory ligand B7x has not been published, knockout of B7x reduced the number of tumor-resident infiltrating Tregs (Abadi et al., 2013). Conversely, administration of B7x-Ig proteins promoted the function and expansion of Tregs in the central nervous system (CNS) in an EAE mouse model (Podojil et al., 2013). Co-inhibitory molecule Tim-3 (T cell immunoglobulin mucin domain-3) was found expressed in a subset of $Foxp3^+$ Tregs and the expression of Tim-3 was associated with higher Treg suppressor functions (Gupta et al., 2012; Sakuishi et al., 2013). Similarly, expression of co-inhibitory molecule TIGIT (T cell Ig and ITIM domain) on Tregs enhanced the suppressive phenotype of Tregs in tumor tissues (Kurtulus et al., 2015). The co-stimulatory receptor ICOS (inducible co-stimulatory molecule) promotes the expansion of Tregs through interaction with ICOS ligand, which further inhibits tumor immunity (Conrad et al., 2012; Faget et al., 2012; Martin-Orozco et al., 2010).

In addition to members of IgSF mentioned in the last paragraph, members of the TNFSF/TNFRSF also modulate Treg homeostasis and function. The co-stimulatory receptor CD27 interacts with its ligand CD70 to promote the expansion of Tregs through reducing the apoptosis of Tregs and inducing IL-2 production from Teffs, which further reduce the adaptive T cell response against tumors (Claus et al., 2012). The co-stimulatory receptor CD30 is critical for Treg-mediated suppression, as CD30-deficient Tregs exhibited significantly compromised effect in preventing GVHD (Dai et al., 2004; Zeiser et al., 2007). The co-stimulatory receptor CD40 is important for the expansion of Tregs, as stimulation of CD40 by soluble CD40L (CD40 ligand) significantly increased the expansion of Tregs *in vitro* and abrogation of CD40/CD40L interaction by blocking antibody or CD40 knockout impaired Treg expansion (Huang et al., 2012; Pan et al., 2010). Receptor 4-1BB is quickly upregulated in activated $CD4^+CD25^+$ Tregs and stimulation of 4-1BB by 4-1BBL (4-1BB ligand) can dramatically expand the Tregs *in vitro* (Schoenbrunn et al., 2012; Elpek et al., 2007). Treatment of mice with agonistic mAbs against co-stimulatory receptor DR3 (death receptor 3) leads to dramatic expansion of Tregs but not Teffs, suggesting a role of DR3 in stimulating Treg proliferation (Schreiber & Podack, 2013). HVEM (herpesvirus entry mediator) expressed on Tregs can interact with gD (glycoprotein D) of HSV-1 (herpes simplex virus-1) to promote Treg proliferation and activation. Additionally, HVEM is also able to interact with IgSF member BTLA to enhance the suppression of Teffs conferred by Tregs (Pasero et al., 2009; Sharma et al., 2014; Tao et al., 2008).

Concluding remarks

With the emerging promise of co-inhibitory molecule (immune checkpoint) inhibitors in cancer immunotherapy, this area has become a hotbed of activity, with enormous efforts focused on the discovery of new receptors and ligands, and the development of new strategies for targeting these molecules (Assal et al., 2015; Ohaegbulam et al., 2015). There is convincing evidence that some of these checkpoint inhibitors also have therapeutically relevant effects on Tregs. Anti-CTLA-4 mAbs with the IgG2a isotype showed enhanced ability compared with IgG2b and IgG1 versions in antitumor activity by mediating a rapid depletion of Tregs and concomitant activation of Teffs at tumor sites (Selby et al., 2013). Two other studies also indicated Fc-γ-dependent elimination of Tregs improved the

efficacy of anti-CTLA-4 mAbs–mediated antitumor immunity in mice (Bulliard et al., 2013; Simpson et al., 2013). Consistent with these reports, in cancer patients treated with Ipilimumab, the clinical benefits were correlated with the ratio of Teffs to Tregs (Hodi et al., 2008; Liakou et al., 2008).

Reducing the suppressive environment conferred by Tregs is an important strategy in cancer immunotherapy (Kim et al., 2014). GITR and OX40 are attractive targets for compromising Tregs, as they are both co-stimulatory receptors to augment the activity of Teffs, whereas at the same time they are expressed constitutively on Tregs to inhibit the suppressive function of Tregs in tumor environment. One group showed that by activation of GITR with agonist mAbs, Tregs became less effective at suppressing Teffs, which led to effective antitumor immunity (Ko et al., 2005; Shimizu et al., 2002). Agonist anti-OX40 mAbs has also been demonstrated to effectively impair suppressive function of Tregs (Hirschhorn-Cymerman et al., 2009; Piconese et al., 2008), and targeting GITR and OX40 in clinical trials.

Combination immunotherapies aim to target multiple co-stimulatory or co-inhibitory molecules to augment the antitumor activity of Teffs while reducing the immune suppressive features of the tumor microenvironment. With the realization of the distinct effects of some co-stimulatory and co-inhibitory molecules on Teffs and Tregs, more comprehensive mechanistic considerations must be taken into account when designing novel immunotherapeutic strategies to treat malignancies. Nevertheless, blocking co-inhibitory molecules CTLA-4, PD-1/PD-L1 axis, B7x, and other checkpoint molecules abrogates the co-inhibitory signals to Teffs, at the same time also suppresses the function of Tregs. Combination immunotherapy using the checkpoint inhibitors in combination with reagents selectively targeting Tregs can possibly improve treatment benefits. Indeed, selective depletion of Tregs by anti-CTLA-4 mAbs did augment the antitumor immunity (Bulliard et al., 2013; Selby et al., 2013; Simpson et al., 2013). In summary, understanding of the impact of co-stimulatory and co-inhibitory molecules on Tregs will assist the development of new strategies targeting Tregs to complement the combination cancer immunotherapies.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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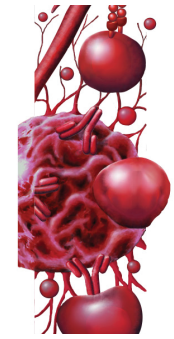
Review

Immunotherapy



Immune checkpoint blockade in human cancer therapy: lung cancer and hematologic malignancies

Tumor immune evasion is one of the hallmarks of cancer, and expression of the B7 family of immune checkpoints (PD-L1, PD-L2, B7-H3, B7x and HHLA2) is one mechanism of immune evasion by tumors to suppress T-cell function. Antibodies blocking these interactions of B7–1/B7–2/CTLA-4 and PD-L1/PD-L2/ PD-1 have had remarkable clinical success in several cancers and are less toxic than traditional chemotherapy. Even though only a small proportion of patients respond to checkpoint blockade, the duration of such responders due to immunological memory is remarkable and is longer than would be expected with any other agent in refractory disease. In this article, we review the therapeutic trials of blocking these pathways in human lung cancer and hematological malignancies.

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Immune checkpoints

Tumor immune evasion is considered one of the hallmarks of malignancy and represents an important step in the evolution of the tumor [1]. The immune system prevents and controls malignancies through immunosurveillance which has been broken down into a three-step process called immunoediting – elimination, equilibrium and escape [2]. Different cancers have different mechanisms to avoid immunosurveillance but one common mechanism is through expression of immune checkpoints. Cancer evolves through genetic and epigenetic instability which leads to failure of conventional chemotherapy and targeted therapy to driver mutations in most patients with metastases. Stimulation of T cells and utilizing T cells to treat cancer is attractive and has advantages: specificity of T cells to a particular antigen; T-cell memory which enables persistent clearance unlike drugs or chemotherapy; and adaptability – the T cell through somatic mutations can generate as many as 10^{15} TCRs (T-cell receptors) and hence can accommodate tumor heterogeneity and tumor evolution [3,4]. Neoantigenic epitopes arise due to somatic mutations in the cancer cells and these antigenic epitopes are normally not expressed in the human genome and play an important role in T-cell immune response. It has been shown that the nonsynonymous mutational burden in non-small-cell lung cancer (NSCLC) correlates with a higher neoantigen burden which in turn is associated with a better response to immune checkpoint blockade [5]. This shows that the genomic landscape of the tumors is important in T-cell response to tumors. In addition to the MHC-peptide/TCR signaling, T-cell-mediated adaptive immune response is regulated by positive costimulation and negative coinhibition from the interaction between the B7 family and their receptor CD28 family. Tumors

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hijack these mechanisms by delivering coinhibitory signals to T cells through expressing coinhibitory ligands on tumor cells such as PD-L1, PD-L2, B7-H3, B7x (B7-H4/B7S1) and HHLA2. The B7–1/B7–2/CTLA-4 and the PD-L1/PD-L2/PD-1 coinhibitory pathways are the furthest along in clinical development, and therapeutic blockade of these pathways has yielded dramatic clinical benefit in melanoma [6–8] and other tumors. Herein, we discuss the clinical trials of immune checkpoint inhibitors in lung cancer and in hematological malignancies.

Immune checkpoint blockade in lung cancer

Blocking antibodies against CTLA-4

Ipilimumab (MDX-010, Bristol-Myers Squibb, Princeton, NJ, USA) is a fully human monoclonal antibody of the IgG1 isotype which binds to CTLA-4. It was first approved based on a Phase III trial, which showed improved overall survival (OS) for the treatment of unresectable or metastatic melanoma in both treatment-naive and previously treated patients [6]. Preclinical models show that taxanes and platinum chemotherapy released tumor antigens and sensitized tumor cells to lymphocyte-mediated killing and hence ipilimumab was tested with this combination in lung cancer [9].

Ipilimumab was tested in a randomized Phase II study in treatment-naive NSCLC patient in combination with carboplatin and paclitaxel in 1:1:1 fashion [10]. Two different modes of administration of ipilimumab were used in the study. In Arm A (concurrent arm) patient received four doses of ipilimumab (10 mg/kg) plus paclitaxel and carboplatin (175 mg/m^2) followed by two doses of placebo plus paclitaxel and carboplatin. Arm B (phased arm) patients received two doses of placebo plus paclitaxel and carboplatin followed by four doses of ipilimumab plus paclitaxel and carboplatin. In the control arm, patient received up to six doses of placebo plus paclitaxel and carboplatin. The eligible patient continued ipilimumab or placebo every 12 weeks as maintenance therapy. This study utilized immune-related progression-free survival (irPFS) as the primary end point [11]. The study met its primary endpoint of irPFS with phased arm (HR: 0.72; p = 0.05), but not with concurrent arm (HR: 0.81; p = 0.13). The phased ipilimumab, concurrent ipilimumab and control arms were associated with a median irPFS of 5.7, 5.5 and 4.6 months, and a median OS of 12.2, 9.7 and 8.3 months, respectively (Table 1) [6]. The rate of grade 3/4 immune-related adverse events was high in the concurrent arm at 20 vs 15% in the phased arm. A nonpreplanned subgroup analysis based on histology showed improved HR of 0.55 for squamous vs 0.82 for nonsquamous (NSCLC) histology. The reason why the phased treatment was superior to concurrent approach was not entirely clear. One hypothesis is that the chemotherapy phased prior to immunotherapy may facilitate immunogenic 'cell death' and lead to improved T-cell priming and better immune responses.

Based on the above study, two Phase III trials are being conducted: NCT01285609, in which patients with squamous NSCLC will be randomized in a 1:1 fashion to carboplatin/paclitaxel/ipilimumab versus carboplatin/paclitaxel alone. The ipilimumab will be given in a phased fashion which will be after two doses of carboplatin and paclitaxel; NCT01450761 being conducted in small-cell lung cancer patients in which patients will be randomized in 1:1 fashion to carboplatin/etoposide/ipilimumab versus carboplatin/etoposide/placebo. Ipilimumab will be infused once every 3 weeks for four doses, then every 12 weeks, until progression of disease or unacceptable toxicity or until the maximum treatment period of 3 years is reached.

Tremelimumab (CP-675,20, AstraZeneca, London, UK) is a human IgG2 monoclonal antibody against CTLA-4 [12]. In a randomized Phase II study of tremelimumab 15 mg/kg every 90 days versus best supportive care in advanced NSCLC patients who had progressed on or after platinum-based chemotherapy, tremelimumab did not improve PFS, but there was a 4.8% relapsed refractory (RR) in the experimental arm [13]. Currently, this drug is being investigated in combination with other anti-PD-1 and anti-PD-L1 antibodies.

Blocking antibodies against PD-1 or PD-L1

To better understand which group of tumors may respond to PD-1/PD-L1 blockade, a simplified model has been proposed to classify tumor microenvironment into four subtypes based on their PD-L1 expression and tumor-infiltrating lymphocyte (TIL) status: type I tumors with PD-L1+TIL+, suggesting adaptive immune resistance and could respond to single agent of PD-1/PD-L1 inhibitors; type II tumors with PD-L1-TIL-, implicating immunological ignorance. This type of tumor may require combination treatment to bring T cells to tumor, such as combining CTLA-4 and PD-1 blockade; type III tumors with PD-L1+TIL-, indicating intrinsic induction of PD-L1 by an oncogenic pathway and the strategy would be to induce T-cell priming and response; type IV tumors with PD-L1-TIL+, suggesting immune tolerance with non-PD-1/PD-L1 suppressors which may need targeting alternative immune checkpoints or signaling pathways [14]. In advanced melanoma, 38%, 41%, 1% and 20% of patients can be categorized to type I, II, III and IV, respectively. In a recent study of tumor lymphocyte infiltration in resected NSCLC, similar to

Table 1. Clinical trials of immune checkpoint inhibitors in non-small-cell lung cancer.

Trial number	Regimen evaluated	Disease/population	Number of patients	Results
NCT00527735	Ipi + carboplatin + paclitaxel (Phase II)	Treatment-naive NSCLC	204	PFS primary end point met in phased ipi arm. Phased Ipi: 5.7 mo (p = 0.05) Concurrent Ipi: 5.5 mo vs Control arm: 4.7 mo irBORR 32% in phased ipi arm Grade 3/4 SAE: 15% in phased Ipi arm
NCT00312975	Tremelimumab vs BSC Phase II	Refractory patients with NSCLC greater than four line of prior treatment	87	ORR: 4.8%
NCT01642004	Nivolumab vs docetaxel Phase III	PD after platinum-based treatment with squamous histology	272	OS: Nivolumab: 9.2 mo (p < 0.001) vs Docetaxel: 6 mo 42% alive at 1 year in nivolumab arm vs 24% in docetaxel arm ORR: 20% in nivolumab (p = 0.008) SAE: pneumonitis 5% in nivolumab arm
NCT01673867	Nivolumab vs docetaxel or pemetrexed (Phase III)	PD after platinum-based treatment with nonsquamous histology	582	OS: Nivolumab:12.2 mos (p = 0.002) vs Docetaxel: 9.4 mo >50% alive at year 1 in nivolumab arm ORR: 19% in nivolumab (p = 0.02) Pneumonitis: 3% Grade 3 or higher SAE: Nivolumab: 10% vs Docetaxel: 54%
NCT01295827	Pembrolizumab (Phase I)	Multiple NSCLC cohorts of treatment-naive as well as previously treated patients	495	OS: 12 mo ORR: 19.4% SD: 21.8% ORR in >50% PD-L1+ : 45.2% Grade 3 or higher SAE: 9.5% Hypothyroidism: 6.9% Pneumonitis: 1.8%
NCT01903993	Atezolizumab vs docetaxel (Phase II)	PD after platinum-based treatment in NSCLC	287	OS: Atezolizumab: 12.6 mo (p = 0.04) vs Docetaxel: 9.7 mo ORR: Atezolizumab: 38% vs Docetaxel: 13%
NCT01633970	Atezolizumab vs platinum-based doublet (Phase Ib)	Treatment-naive NSCLC	37	ORR: 67%
NCT01693562	Durvalumab (Phase I/II)	PD after platinum-based treatment in NSCLC	198	ORR: 14%, Grade 3 or higher SAE: 6%

AEs: Adverse events; BSC: Best supportive care; Ipi: Ipilimumab; irBORR: Immune-related best overall response rate; mo: Months; NSCLC: Non-small-cell lung cancer; ORR: Overall response rate; OS: Overall survival; PD: Progressive disease; PFS: Progression-free survival; SAE: Serious adverse events; SD: Stable disease.

breast cancer, an intense tumor lymphocytic infiltration (>50%) was seen in 11% of patients and was associated with an improved OS (OS: HR: 0.45; 95% CI: 0.23–0.85; p = 0.01) [15]. Hence strategies utilizing the immune profile of the tumor microenvironment can be used to tailor PD-1/PD-L1-based immunotherapy and to design future trials in lung cancer.

Nivolumab (a human IgG4 anti-PD-1 mAb, BMS-936558, MDX-1106, Bristol-Myers Squibb) has been approved by US FDA to treat advanced metastatic NSCLC patients who have progressed on or after platinum therapy based on two trials [16,17]. In Checkmate 017 trial, patients with squamous cell lung cancer were randomized 1:1 to nivolumab, at a dose of 3 mg/kg every 2 weeks, or docetaxel, at a dose of 75 mg/m^2 every 3 weeks [16]. The median OS was 9.2 months with nivolumab versus 6.0 months with docetaxel. The overall response rate (ORR) was 20% with nivolumab versus 9% with docetaxel (p = 0.008). Treatment-related adverse events (AEs) were seen in 7% of patients getting nivolumab versus 55% of patients getting docetaxel. PD-L1 expression was not found to be predictive or prognostic of response or benefit.

Checkmate 057 trial was a global randomized Phase III trial comparing patients with advance stage nonsquamous NSCLC [17], who have progressed on and after platinum-based therapy/tyrosine kinase inhibitors to nivolumab 3 mg/kg every 2 weeks or docetaxel at a dose of 75 mg/m^2 every 3 weeks. Patients randomized to nivolumab had a median OS of 12.2 months (ORR = 19%) versus 9.4 months in the docetaxel arm (ORR = 12%) with 27% reduction in risk of death. 10% of people in the nivolumab arm had grade 3/4 events versus 54% in docetaxel group. In this study, tumor PD-L1 positivity was predictive of benefit to nivolumab. The approval of these drugs represents a major advance, as most lung cancer patients are older and the favorable safety profile of the checkpoint inhibitors makes this very attractive.

Pembrolizumab (anti-PD-1 monoclonal antibody, MK 3475, Merck, Kenilworth, NJ, USA) is a humanized IgG4 anti-PD-1 monoclonal antibody. It was recently granted accelerated approval by FDA based on a large Phase I study KEYNOTE-001, in which 495 patients were treated at doses of 2 or 10 mg/kg every 3 weeks or 10 mg/kg every 2 weeks [18]. The primary endpoint of the study was safety and efficacy of pembrolizumab. Both treatment-naive and patients who have progressed on or after platinum therapy were eligible. The ORR was 19.4% and the median duration of response was 12.5 months. The response rate and duration of response were higher in treatment-naive patients (ORR: 24.8%; 23.3 months) compared with previously treated patients (ORR: 18.0%; 10.4 months). The ORR did not differ by dose, schedule or histology. Current or former smokers had higher response rates (22.5 vs 10.3%) than nonsmokers. Membranous PD-L1 expression in at least 50% of cells was selected as a biomarker cutoff based on the ease of use and receiver operating curve analysis. Tumors with tumor PD-L1 positivity of >50, 1–49 and <1% had ORR of 33, 17 and 3% respectively. 10% of patients had grade 3 or higher side effects; hypothyroidism was the most common immune-related side effect seen in 34 patients (6.9%) followed by pneumonitis in 18 patients (3.6%). Based on these results pembrolizumab is indicated for metastatic NSCLC whose tumors express PD-L1 after progression on platinum-based chemotherapy. Further, Phase II/III confirmatory trials building on these results are ongoing with pembrolizumab in NSCLC.

Atezolizumab MPDL3280A (Genentech/Roche, CA, USA) is an engineered human IgG1 monoclonal antibody (mAb) that targets PD-L1 [19]. This antibody lacks an Fc component, thus avoiding antibody-dependent cytotoxic cellular killing of bystander immune cells. Atezolizumab was studied in multicenter open-label randomized Phase II study (POPLAR; NCT01903993) in which it was compared with docetaxel 75 mg /m^2 in advanced or metastatic NSCLC patients who have progressed on platinum-based therapy [20]. These patients were stratified by histology, PD-L1 status (high, any and no expressors) and prior treatment. The primary outcome was on efficacy, safety and predictive biomarkers. A total of 287 patients were randomized and the ORR was 38% in atezolizumab arm compared with 13% with docetaxel arm. OS was significantly improved in the atezolizumab arm 12.6–9.7 months in the docetaxel (HR: 0.73; p = 0.04). Patients who have the highest expression of PD-L-1 in their tumor derived the most benefit with 41% improvement compared with nonexpressors. Despite longer duration of treatment with atezolizumab (3.7 vs 2.1 months), more patients in docetaxel arm had withdrawals due to treatment-related adverse effect (22 vs 8%) [21]. Atezolizumab has also been studied in the first-line setting in combination with platinum therapy. This Phase Ib study evaluated atezolizumab with a wide variety of platinum doublets [22]. The combination was well-tolerated and the most common atezolizumab-related grade 3-4 serious AEs were anemia, neutropenia and thrombocytopenia. No pneumonitis was seen. There was one atezolizumab-related death due to candidemia after prolonged neutropenia. The ORR across all arms was 67%. These responses were seen in each arm independent of PD-L1 expression.

Durvalumab (MEDI4736, Medimmune, MD, USA) is a fully humanized IgG1 mAb that blocks PD-L1 binding to PD-1 and CD80, leading to enhanced tumor killing by reducing T-cell inhibition [23]. The antibody has a high affinity and specificity to only for PD-L1 and does not bind to PD-L2. In a Phase I dose escalation/expansion study in NSCLC patients, 16% had responded to durvalumab administered 10 mg/kg every 2 weeks. ORR was higher in squamous histology at 21% compared with 10% in nonsquamous NSCLC. The drug was well-tolerated and grade 3–4 AEs were seen in 6% of study population [24]. Currently, both the PD-L1 inhibitors have progressed to Phase III trials in NSCLC (Table 2).

PD-L1 positivity as a biomarker in NSCLC

As noticed from above studies, the response rates of anti-PD-1/PD-L1 antibodies in NSCLC as a single agent are around 20% in unselected patient population. Thus a significant proportion of patients do not derive clinical benefits. It is paramount to identify predictive biomarkers to select appropriate patient population who may or may not benefit from immunotherapy and also to guide future trial designs.

Table 2. Ongoing clinical trials with immune checkpoint inhibitors in non-small-cell lung cancer.

Clinical trial identifier	Phase	Experimental regimen	Population	Primary end points
CTLA-4 antibodies				
NCT01285609	III	Ipilimumab + paclitaxel + carboplatin	Second-line metastatic squamous NSCLC	OS
NCT01450761	III	Ipilimumab + etoposide/platinum	First-line metastatic SCLC	OS
NCT01998126	1B	Ipilimumab + erlotinib/crizotinib	First-line EGFR or ALK (anaplastic lymphoma kinase)-mutated NSCLC	DLT
NCT02477826	III	Nivolumab vs nivolumab + ipilimumab, vs nivolumab + platinum doublet vs platinum doublet chemotherapy	First-line metastatic NSCLC	OS PFS
PD-1 antibodies				
NCT02259621	I	Neoadjuvant nivolumab	Neoadjuvant Stage 1–3 (N1 disease)	DLT
NCT02393625	I	Nivolumab + ceritinib	ALK-positive metastatic lung cancer	DLTw ORR
NCT02343952	II	Pembrolizumab	Unresectable IIIA/IIIB NSCLC after receiving concurrent chemoradiation	Event (death) rate
NCT02564380	II	Pembrolizumab maintenance vs placebo	First-line metastatic setting following platinum-based doublet metastatic squamous NSCLC	PFS
NCT02142738 (Keynote- 024)	III	Pembrolizumab vs carboplatin + paclitaxel	First-line metastatic setting in strong PDL1 expressing NSCLC	PFS
NCT02578680 (KEYNOTE-189)	III	Pembrolizumab + platinum + pemetrexed vs placebo+ platinum + pemetrexed	First-line metastatic setting Nonsquamous NSCLC	PFS
NCT02220894 (Keynote 042)	III	Pembrolizumab vs platinum-based chemotherapy	First-line metastatic setting in strong PDL1 expressing NSCLC	OS
NCT02316002	II	Pembrolizumab	Resected oligometastatic NSCLC	PFS
NCT02504372 (Keynote -091) (PEARLS)	III	Pembrolizumab vs placebo	Resected stage 1b-IIIA NSCLC after standard adjuvant therapy	DFS
PD-L1 antibodies				
NCT02409342 (IMpower110)	III	Atezolizumab + pemetrexed + cisplatin or carboplatin	First-line metastatic nonsquamous NSCLC	PFS
NCT02366143 (IMpower150)	III	Atezolizumab + carboplatin + paclitaxel + bevacizumab	First-line metastatic nonsquamous NSCLC	PFS
NCT02367794	III	Atezolizumab + carboplatin + paclitaxel or nab-paclitaxel	First-line metastatic squamous NSCLC	PFS
NCT02367781	III	Atezolizumab + carboplatin + nab-paclitaxel	First-line metastatic nonsquamous NSCLC	PFS
NCT02125461 (PACIFIC)	III	Durvalumab vs placebo	NSCLC post platinum-based concurrent chemoradiation therapy	OS
NCT02453282 (MYSTIC)	III	Durvalumab + tremelimumab vs durvalumab vs platinum-based therapy	First-line metastatic NSCLC	PFS
NCT02542293	III	Durvalumab + tremelimumab vs platinum-based therapy	First-line metastatic NSCLC	OS
NCT02273375	III	Durvalumab vs placebo	Resected stage 1b-IIIA NSCLC after standard adjuvant therapy	DFS
NCT02352948	III	Durvalumab vs durvalumab + tremelimumab	Third-line metastatic NSCLC	OS

DFS: Disease-free survival; DLT: Dose-limiting toxicity; NSCLC: Non-small-cell lung cancer; ORR: Overall response rate; OS: Overall survival; PFS: Progression-free survival; SCLC: Small-cell-lung cancer.

Expression of PD-L1 in tumor cells has been used and recommended as a biomarker. Indeed, the FDA approval of pembrolizumab comes with a companion diagnostic test, the PD-L1 IHC 22C3 pharmDx assay to select PD-L1 positive lung cancer patients, based on the superior efficacy observed in this subset of patients. On the other hand, nivolumab has been approved without the use of companion diagnostic test and the CHECKMATE-017 study showed no association between PD-L1 expression despite showing high correlation between PDL-1 expression and tumor response in the KEYNOTE-001 study. How to explain these discrepant results? More accurate assessment of the immune environment is important and hence TILs, immune signature showing the degree of activation of the immune system, presence of other immune checkpoint ligands needs to be assessed in conjunction with PD-L1; factors intrinsic to PD-L1 measurement – the use of different antibodies to test the expression of PD-L1, the use of different types of pathology specimen and sites for staining and what constitutes positivity need to be standardized.

Immune checkpoint blockade in hematological malignancies

Hodgkin lymphoma

Hodgkin lymphoma (HL) is a B-cell lymphoma, and relapsed HL has poor survival. In HL, the PD-1/PD-L1 pathway is amplified through three mechanisms. First, 9p24.1 amplification is a recurrent genetic abnormality and this region encodes for the PD-L1 and PD-L2, which are hence amplified. Second, 9p amplification also encodes the JAK/STAT pathway which results in overexpression of PD-L1. Third, HL has a high expression of EBV-related proteins which in turn increases PD-1 expression [25]. Nivolumab was studied in relapsed refractory HL at a dose of 3 mg/kg every 2 weeks. 23 patients were enrolled and 78% had previous autologous stem cell transplant (autologous HSCT; hematopoietic stem cell transplant) and brentuximab vedotin (anti-CD30 mAb) and hence these patients are considered highly refractory [26]. The ORR was 87%, 17% with a complete response (CR), 70% with a partial response and 13% had stable disease and the PFS at 6 months was 86% (Table 3). Tumor tissue showed copy number gains and increased protein expression of PD-L1 and PD-L2. Pembrolizumab was studied in the KEYNOTE-013 trial in relapsed refractory HL and similar to the previous trial most failed autologous HSCT and all failed brentuximab [27]. The ORR was 65% with 16% achieving CR and the median duration of response was not yet reached. A significant increase in the absolute number of circulating total lymphocytes, T cells (CD4 and CD8 subsets) and natural killer (NK) cells was seen post treatment. RNA profiling of pre- and post-treatment blood samples showed that the ten-gene IFN-γ-induced signature, the 18-gene expanded immune signature and the 13-gene TCR signature were all significantly upregulated. These show that enhanced T-cell specific and possibly NK-cell immunity is increased post anti-PD-1 treatment. These studies showed that PD-1 blockade resulted in impressive response rates in HL and significant toxicities were far less when compared with chemotherapy, and immune-related AEs were observed in <20% of those treated. Hence further studies are ongoing in HL as a single agent and in combination with chemotherapy and CTLA-4 inhibitors.

Non-Hodgkin lymphoma

Non-Hodgkin lymphoma (NHL) encompasses a wide variety of lymphomas and based on the cell of origin can be classified as B cell and T cell or based on prognosis as indolent and aggressive. PD-L1 protein is expressed in the tumor cells of various NHL types namely – primary mediastinal large B-cell lymphoma, T-cell/histiocyte-rich B-cell lymphoma, EBV-positive and -negative post-transplant lymphoproliferative disorder, EBV-associated diffuse large B-cell lymphoma, plasmablastic lymphoma, extranodal NK/T-cell lymphoma, nasopharyngeal carcinoma and HHV8-associated primary effusion lymphoma [28,29].

The CTLA-4 inhibitor ipilimumab was tested in relapsed refractory NHL and the ORR was disappointing at 11% (2/18) but the responses in the two patients lasted for >31 and 19 months, which is generally longer than would be expected in relapsed refractory disease [30]. T-cell proliferation to recall antigens was also increased as would be expected. Pidilizumab, a PD-1 blocking antibody was tried in relapsed refractory lymphoma and was given as a single dose with an ORR of 33% and no dose-limiting toxicity was identified, thus providing evidence that PD-1 blockade is safe in hematological malignancies [31]. A sustained increase in $CD4^+$ lymphocytes was seen in the peripheral blood in those treated. Pidilizumab was then combined with rituximab (anti-CD20 Ab) in a Phase II trial for rituximab-sensitive relapsed follicular lymphoma [32]. Pidilizumab also increases NK-cell activity and hence this combination was designed to increase the NK-cell-mediated ADCC activity of rituximab. There were no autoimmune or immune-related AEs of grade 3/4 and the ORR was 66% which suggested activity of this combination, but the population of rituximab sensitive relapse makes it difficult to interpret the true value of this combination [25,33]. Nivolumab was also tested in 29 patients with relapsed refractory NHL at 3 mg/kg

Table 3. Clinical trials of immune checkpoint inhibitors in hematological malignancies.

Trial number	Agent	Disease/ population	Number of patients	Results and notes
NCT01592370	Nivolumab 3 mg/kg every 2 weeks	RR Hodgkin lymphoma	23	ORR: 87%, CR: 17%, PR: 70%, SD: 13% Copy number gains in PD-L1 and PD-L2 was present
NCT01953692 (KEYNOTE 013)	Pembrolizumab 10 mg/kg every 2 weeks	RR Hodgkin lymphoma	15	ORR: 53%, CR: 20%, PR: 33% SAE – respiratory events (20%), thyroid events (20%)
NCT00089076	Ipilimumab 3 mg/kg	RR NHL	18	Two patients with clinical responses lasting 31 and 19 months T-cell proliferation to recall antigens was significantly increased
–	Pidilizumab (CT-011)	Advanced hematological malignancies	17	Cumulative survival at 21 days – 76%, 1 CR (FL). Sustained elevation in peripheral blood $CD4^+$ lymphocytes was present
NCT00904722	Pidilizumab Rituximab	Rituximab sensitive relapsed Follicular lymphoma	29	ORR: 66%, CR: 52%, Median PFS: 18.8 months Expression of activating receptor NKG2D on NK cells was significantly increased
NCT01592370	Nivolumab 1 or 3 mg/kg every 2 weeks for 2 years	RR hematological malignancies	27	ORR by subtypes – DLBCL: 36%, FL: 40%, T-NHL: 17%, PTCL: 40% SAE – pneumonitis: 7%
NCT00532259	Pidilizumab	Post Auto HSCT	66	PFS at 16 week follow-up: 0.72 (met study end point) ORR – 51%. Treatment was associated with an increase in circulating lymphocytes
Ongoing clinical trials with interim results				
MC1485	Pembrolizumab	Relapsed refractory CLL, Richter's	16	4/5 Richter's patients responded 2/2 CLL had stable disease
NCT02289222	Pembrolizumab Pomalidomide Dexamethasone	RR MM	22/16 weeks	ORR: 50%
NCT01953692	Pembrolizumab	RR PMBCL	9/20 weeks	ORR: 49%
NCT02077959	Pidilizumab + lenalidomide	RR MM	12	ORR: 66.6%
NCT01067287	Pidilizumab + dendritic cell vaccine	Post autologous HSCT	22	Three CR's after immunotherapy
NCT02036502	Pembrolizumab Lenalidomide Dexamethasone	RR MM	17/41 weeks	ORR: 76%

CLL: Chronic lymphocytic leukemia; CR: Complete response; DLBCL: Diffuse large B cell lymphoma; FL: Follicular lymphoma; HSCT: Hematopoietic stem cell transplant; MM: Multiple myeloma; NHL: Non-Hodgkin Lymphoma; T-NHL: T-cell non-Hodgkin lymphoma; NK: Natural killer; ORR: Overall response rate; PFS: Progression-free survival; PMBCL: Primary mediastinal B cell lymphoma; PR: Partial response; PTCL: Peripheral T-cell lymphoma; RR: Relapsed refractory; SAE: Serious adverse events; SD: Stable disease.

every 2 weeks. The ORR was 36%, but varied according to the subtype – diffuse large B-cell lymphoma, follicular lymphoma and peripheral T-cell lymphoma having an ORR of 36, 40 and 40%, respectively [34]. Hence these studies show that checkpoint inhibition is feasible in a select subgroup of NHL and this could vary by histology or the molecular pathways involved in B-cell transformation may influence the response.

Autologous HSCT

Autologous HSCT (ASCT) is performed in NHL as a method of consolidation in NHL or for relapsed refractory disease and the time period of immune reconstitution could be a good window to augment T-cell response. Pidilizumab was tested in post autologous HSCT in aggressive non-Hodgkin lymphoma in those who had chemo-sensitive disease [35]. Pidilizumab was

given at 1.5 mg/kg every 42 days, 30–90 days from ASCT during the time of immune reconstitution. The PFS at 16 months was 0.7 (0.51–0.82) while the threshold value was 69% to warrant further study. This study met its primary end point and further randomized studies (NCT02362997) are being done to see whether PD-1 inhibitors can improve disease-free survival after autologous HSCT.

Allogeneic HSCT

Allogeneic HSCT is used as a treatment option for treating relapsed refractory lymphomas. Two concerns remain: is allogeneic HSCT safe after PD-1 inhibitor treatment? Second, exacerbation of acute or chronic graft versus host disease (GVHD) after allogeneic HSCT. Mouse models show that PD-1/PD-L1 interactions dampen acute GVHD while it increases chronic GVHD [36]. Retrospective analyses of allogeneic HSCT after PD-1 inhibitors show that the disease-free survival was higher than would be expected and similarly a higher than expected GVHD and veno-occlusive disease was also seen [37]. Ipilimumab was tested postallogeneic HSCT at dose cohorts between 0.1 and 3 mg/kg [38]. Twenty-nine patients with hematological malignancies which relapsed after allogeneic HSCT were included in three dose cohorts. Dose-limiting toxicity was not encountered and, more importantly, there was no GVHD or graft rejection and immune-related AEs were acceptable. Notably, three patients with Hodgkin disease and one patient with refractory mantle cell lymphoma had a response. This is an important trial to show that checkpoint blockade was feasible in patients with hematological malignancies after allogeneic HSCT without causing GVHD or graft rejection.

Multiple myeloma & leukemia

Nivolumab was also tested in a dose escalation trial in persons with relapsed refractory hematological malignancies and was administered every 2 weeks for 2 years. Interestingly, the stable disease rate in multiple myeloma was 63% with a median duration of follow-up of 62 weeks [39]. Hence there are multiple ongoing trials with PD-1 inhibitor as single agent (NCT01953692) or in combination with immunomodulators (Imids) (NCT02036502, NCT02289222) and dendritic cell vaccines (NCT01067287) in myeloma.

In myeloid malignancies, aberrant expression of PD-L1 was noted in myelodysplastic syndrom (MDS), chronic myelomonocytic leukaemia (CMML) and acute myeloid leukaemia (AML) in 34, 14 and 15% of patient samples tested. Moreover, PD-1 and PD-L1 is highly expressed in MDS blasts after treatment with hypomethylating agents [40], and hence this is being currently tested in a Phase I trial of an anti-PD-1 antibody (MEDI 4736) in combination with azacitidine in this disease. The KEYNOTE-013 trial is exploring pembrolizumab in a multicohort trial (NCT01953692) and patients will be included in different cohorts based on lymphoma subtype and PD-L1 positivity. In CML, PD-L1 is expressed on the leukemic cells and PD-1 is expressed on tumor-specific cytotoxic T cells [41], and hence PD-1 inhibitors are being tested in combination with dasatinib (NCT02011945).

Adverse effects & response criteria

The toxicity of these agents is less and manageable when compared with chemotherapy or even some targeted therapy. Adverse events are more immune related and the common AEs are colitis, pneumonitis, thyroiditis, hypophysitis and dermatitis which could be managed with supportive care and steroids. These AEs are less with the PD-1 and PD-L1 inhibitors than the CTLA-4 inhibitors as PD-L1/PD-1 is more involved in the effector phase of the immune function. Comprehensive reviews of immune-related AEs associated with the checkpoint inhibitors have been published [42–44].

Patients who are treated with checkpoint inhibitors can initially have a disease flare before they have a disease response with a median CR of approximately 30 months in the initial trials [45]. In order to account for the time duration and the pattern of responses, an immune-related RECIST criterion has been developed which requires confirmation of progressive disease by a repeat scan after a certain time interval [11]. As we continue to learn more about the pattern of responses these response criteria will continue to be refined.

Conclusion & future perspective

The CTLA-4 and PD-1/PD-L1 blocking antibodies have shown therapeutic success and many other immune checkpoints are being currently evaluated. Antibodies against other checkpoints inhibitors that are furthest in evaluation are LAG-3 (IMP321, Immuntep; BMS-986016, BMS), TIM-3 and VISTA, which are currently being tested in clinical trials. Other members of the B7 family, namely, B7-H3, B7x and HHLA2 are being tested in preclinical models and are soon to enter drug development. Similarly, agonists for costimulatory T-cell ligands, namely, OX40, 41BB (urelumab, BMS-663513) and ICOS are also in clinical development. Combinatorial therapies with the various checkpoint inhibitors, CAR T cells, radiotherapy, immunogenic chemotherapy like anthracyclines, tyrosine kinase inhibitors, fusion vaccines and immunomodulators are also expected to increase response to these therapies. In particular, combination therapies with neoantigen-based vaccines along with immune checkpoint inhibitors can further drive personalized

medicine toward higher tumor specificity. Biomarker profile of responders including TCR repertoire, CD4/CD8 T-cell profile, cytokine signature, immune check point expression in tumor cells, macrophages or T cells are also being developed to identify markers of early response. Imaging criteria of what constitutes response, and whether the modified response criteria can sufficiently predict overall survival and lead to approval of these drugs are yet to be determined. In conclusion, immune checkpoint inhibitors have shown remarkable clinical success and we are likely to see improved success with these agents in the future.

Financial & competing interests' disclosure

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Executive summary

Immune checkpoints

- Tumor immune evasion is one of the hallmarks of cancer and expression of immune checkpoints is an important mechanism of immune evasion.
- The two of major coinhibitory pathways currently known are: the B7–1/B7–2/CTLA4 which is involved in early T-cell activation; and the PD-L1/PD-1 pathway which is involved in peripheral tolerance. Inhibition of these pathways has led to a remarkable therapeutic success in some patients.

Blocking antibodies against PD-1 or PD-L1

- The PD-1 inhibitors nivolumab and pembrolizumab are effective in approximately 20% of patients with non-small-cell lung cancer (NSCLC) and have been currently approved as monotherapy for the treatment of metastatic NSCLC after progression on platinum-based therapy.
- The PD-L1 inhibitors atezolizumab and durvalumab have also shown good activity in Phase I trials of NSCLC and are currently being studied in Phase III trials.

PD-L1 positivity as a biomarker in NSCLC

- Factors which influence response to checkpoint inhibitors include smoking status, PD-L1 expression on tumors, TIL infiltrate and mutational burden of tumors.
- Using PD-L1 expression as a predictive biomarker has significant limitations and hence cannot be recommended for nivolumab.

Immune checkpoint blockade in hematological malignancies

- In hematological malignancies, Hodgkin lymphomas has high response rates to PD-1 inhibitors and are likely to be approved for this indication. In non-Hodgkin lymphoma, the checkpoint inhibitors have limited activity which varies depending on the histology and the B-cell pathway activated.
- An important concept is the safety of immune checkpoint inhibitors pre- and postallogeneic transplant, and early studies indicate that the checkpoint inhibitors are safe although their role in acute and chronic graft versus host disease may be different.

Adverse effects & response criteria

- Check point inhibitors have different response patterns and adverse effects when compared with traditional agents and hence it is important to be aware of the immune-related response criteria and immune-related adverse events to checkpoint inhibitors.

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